MOSQUITO TRANSMISSION OF DOG HEARTWORM (*DIROFILARIA IMMITIS*), FROM THE LABORATORY TO THE FIELD: DNA BARCODING BLOOD MEAL ANALYSIS FOR VECTOR INCRIMINATION, REFINING DEGREE-DAY DEVELOPMENT MODELS FOR DIURNAL TEMPERATURE FLUCTUATION, AND ASSESSING KNOWLEDGE AND SOCIO-BEHAVIORAL RISK FACTORS IN TWO ENDEMIC RESIDENTIAL AREAS.

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COMPREHENSIVE INVESTIGATION OF DOG HEARTWORM TRANSMISSION ECOLOGY: VECTOR FEEDING PATTERNS; MODEL OPTIMIZATION FOR FLUCTUATING TEMPERATURE EFFECTS ON *DIROFILARIA IMMITIS* EXTRINSIC INCUBATION PERIOD; AND KNOWLEDGE, ATTITUDES, AND PRACTICES OF RESIDENTS IN ENDEMIC COMMUNITIES

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Dog heartworm is a mosquito-borne filarial disease found globally that can be potentially fatal to dogs and cats if left untreated. Current control measures rely solely on preventive drugs (macrocyclic lactones) to kill immature larvae in susceptible hosts, or immiticide to eliminate adult heartworms from infected animals; however there is evidence of resistance in wild *D. immitis* populations. Little is known about dog heartworm transmission ecology and how its wide range of potential mosquito vectors contribute to disease maintenance across differing habitats and domestic dog populations. Incriminating key mosquito vectors and identifying socio-behavioral risk factors for transmission would help create informed vector control approaches to manage or eradicate dog heartworm disease. In my research, I have employed and developed effective methods for vector incrimination and surveillance, investigated the effects of temperature fluctuation on *D. immitis* development in mosquito vectors, and conducted door-to-door questionnaires and entomological surveys in endemic communities to determine community knowledge and practices regarding dog heartworm and mosquitoes.

DNA barcoding can be a useful tool for mosquito blood meal analysis, and can reveal vector-host links that are crucial to understanding the potential disease risk from mosquito vector species in an area. To determine blood feeding patterns of mosquito species in Ithaca, NY, I deployed various trapping and collection methods in high human and wildlife traffic areas and performed blood meal analysis on wild-caught specimens. Many DNA barcoding primers and methods are available for this purpose, but success

rates and host identification standards are very rarely reported. To determine the limits of my method I designed a series of quality control analyses using blood samples taken from local wildlife, which were extracted and analyzed individually and as mixed blood samples. I discovered inherent biases of each primer set, and describe for the first time the identification of both hosts in a mixed blood sample from a single DNA barcode query of sequence databases. Recommendations for blood meal analysis projects of different scales are provided as well as implications of the method for disease surveillance and vector incrimination.

D. immitis extrinsic incubation period is predicted by a standard heartworm development unit (HDU) degree-day model that is used to define time periods and geographical areas at risk of dog heartworm transmission. The HDU model is based on average daily temperature above the accepted 14°C threshold for *D. immitis* development in the mosquito vector, but this ignores temperature fluctuation above and below the baseline average temperature. To see if *D. immitis* extrinsic incubation period differs between fluctuating and constant temperature conditions, and to determine the validity of the HDU model, I infected cohorts of Liverpool strain *Ae. aegypti* with *D. immitis* microfilariae and monitored larval development upon dissection. Larval developmental stage was compared between constant and fluctuating treatments of the same average daily temperature, and HDU predictions were assessed against hourly calculations of development unit accumulation. Temperature fluctuation was more accurately modeled by hourly computations of development units than daily calculations, and larvae in fluctuating treatments that spanned the developmental threshold were detected in mosquito heads eight days sooner than larvae at the constant temperature, and they developed sooner than predicted by the standard HDU model. The implication for HDU models predicting transmission period or geographical range is that hourly calculations of heartworm development units should be used for data regarding fluctuating temperature or temperatures approaching the developmental threshold,

otherwise, standard HDU models are likely to underestimate heartworm transmission risk at the beginning and end of the transmission season and in colder regions.

Dog heartworm is primarily a disease of domestic dogs and can cause serious complications in domestic cats, therefore, these animals' well-being relies on their owners' knowledge and practices regarding heartworm prevention and mosquito avoidance. To determine community knowledge regarding dog heartworm, mosquito-borne, disease, and mosquito reduction/avoidance practices, I designed and administered a knowledge, attitudes, and practices questionnaire to residents in two dog heartworm endemic communities. Entomological surveys and continued adult mosquito collections were continued in both neighborhoods after the questionnaires, and the data were analyzed to determine residents' knowledge level, disease concern, and prevention practices. Most residents were not aware that mosquitoes transmit dog heartworm, but it was of high concern to them. Most pet owners had their animals on preventive medication, and owning a dog was associated with correct mosquito knowledge regarding important breeding sites and daily activity. The two most common reasons for pet owners not administering preventive medication to their animals were 1) being unaware of the risk to their pet, and 2) consciously deciding that the risk to their particular pet was low. Recommendations for public health messaging by veterinarians and health officials are to emphasize the risks and cost of heartworm disease treatment in comparison to prevention, highlight the importance of mosquito avoidance and reduction, and to provide detailed information regarding mosquito activity and common garden items that can serve as container-breeding sites.

BIOGRAPHICAL SKETCH

Nicholas Ledesma grew up in New York City, where experiences with nature and wildlife were rare and special to him. This led to a constant influx of insects and arthropods as childhood pets, which surprisingly garnered much support and encouragement from his parents. When not searching for urban fauna under rocks and logs, he scoured library shelves for science and nature books that would come to shape his outlook and future aspirations.

Over his varied experiences including working in a butterfly vivarium, volunteering to help oiled African penguins in South Africa, and handling domestic animals as part of his undergraduate Animal Science major at Cornell University, Nicholas knew he wanted to pursue a career in veterinary medicine, but entomology was a perpetual hobby. It was Dr. Laura Harrington's course that introduced him to the field for which his competing interests were actually complementary: medical and veterinary entomology. He added Entomology as a second major and, with limitless support and motivation from his parents and mentors, he applied and was accepted to Cornell University's combined DVM/PhD program in 2008.

Under Laura's guidance, Nicholas developed his interests and acquired in public health, vector-borne disease ecology, epidemiology, and field surveillance of diseases and their vectors. Nicholas aims to continue his studies in these and related fields during his remaining years of the veterinary curriculum and after completion of his degree.

This dissertation is dedicated to
my mother and Todd, my father and Jan.

Their loving presence and
encouragement have allowed me to
achieve my goals every step of the way.

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My work in Florida is the result of the endurance and dedication of a team of 5 research volunteers: Grace Ahn, Mike Hyon, Chris Leyen, Mark Macapagal, and Andrea Stone. Drs. Phil Kaufman and Rui-De Xue offered us space, traps, and equipment. Anastasia Mosquito Control personnel helped us trap and conduct surveys, and were always a helpful, friendly resource. Columbia County Mosquito Control provided advice and prior collection data. The residents of Lake City and St. Augustine South were welcoming to our door-to-door surveying and their participation in allowing us to collect on their property was invaluable.

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CHAPTER ONE

LITERATURE REVIEW

Mosquito vectors of dog heartworm in the United States: vector status and factors influencing transmission efficiency*

Dog heartworm is obligately transmitted by mosquitoes known as "vectors." Little information is available about the important *D. immitis* vectors in the United States. Below, we summarize the biology and incidence of potential dog heartworm vectors. We begin with an overview of the heartworm life cycle, provide a discussion of currently reported vector species in the United States, present ecological and biological factors that may modulate heartworm vector competence, and present criteria for ranking important vector species. We conclude by proposing that heartworm control would benefit by targeting mosquito vectors, and we suggest ways in which veterinarians can incorporate the recognition of vector importance into heartworm prevention recommendations imparted to clients.

The heartworm life cycle *D. immitis* is a filarial worm causing disease in dogs and heartworm-associated respiratory disease (HARD) in cats (Bowman and Atkins 2009). This parasite also infects coyotes (Sacks et al. 2004), foxes, sea lions, penguins (Sano et al. 2005), and, rarely, humans (Grieve et al. 1983, Simón et al. 2009, Theis 2005).

The complete life cycle of *D. immitis* consists of several developmental stages in both the invertebrate vector and vertebrate hosts. Dogs become infected when third-stage larvae (L3) exit the

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labium of infected mosquitoes during blood feeding. L3 then move across the host skin to enter the mosquito feeding wound. Development from L3 to L4 stage occurs in canine muscles and submuscular membrane at the site of inoculation after approximately 3 days. Between 50 and 58 days post infection, larvae develop to a young adult stage, which enter the bloodstream through the surrounding vasculature and migrate to host pulmonary arteries. The parasite then completes maturation in host lung tissue. Mature *D. immitis* reproduce sexually, and microfilariae released by gravid females into the host's blood are usually seen 6 to 9 months after infection. Microfilariae travel in the peripheral circulation where they can be ingested by mosquitoes in the host blood meal (Grieve et al. 1983, Theis 2005, Knight and Lok 1998).

Once ingested by a mosquito, microfilariae escape the blood bolus in the midgut and enter Malpighian tubule lumina at their junction with the gut. Parasite development to the L2 stage begins after invasion of Malpighian tubule primary cells (Bradley and Nayar 1987, Serrão et al. 2001). On completion of development, the L2 stage larvae exit the Malpighian tubules and migrate through the mosquito body cavity (hemocoel) from the abdomen toward the head. On entering the head, *D. immitis* develop into infectious stage larvae (L3). The infective stage of *D. immitis* exhibits positive thermotaxis (Kartman 1953, Stueben 1954), initiating exit from the mosquito mouthparts on feeding on a warm-blooded host. Emergence from the labium is accompanied by small amounts of mosquito hemolymph, which, together with fluid in the wound, facilitate larval movement across the skin and into the host (Grieve et al. 1983).

The heartworm development rate in mosquitoes is dependent on the number of accumulated thermal units above the parasite development threshold or "heartworm development units" (HDUs) (Bowman and Atkins 2009, Knight and Lok 1998, Lok et al. 1998). Microfilarial development to L3 stage

requires approximately 130 HDUs, which can be accumulated in as little as 8 days at an average daily temperature of 30°C or as long as a month at 18.3°C (Knight and Lok 1998).

Current known vectors of dog heartworm in the United States Published studies on vector competence for dog heartworm include studies of wild mosquitoes and those conducted with potentially inbred laboratory populations. Studies of field collected mosquitoes include direct examination of wild-caught adult females for the presence of filariids or assessment of *D. immitis* development after feeding wild-caught mosquitoes with infected host blood (Scoles et al. 1998). The most important assessment approach for determining important vectors of dog heartworm in nature is field collection of mosquitoes followed by confirmation of infectious parasite stages. This first assessment should then be followed with infection of field-collected mosquitoes (reared from eggs, larvae or pupae) of a uniform age with a uniform dosage of parasite infected blood.

Field surveys of vector populations that do find natural infections of *D. immitis* in mosquitoes often make a useful distinction between the presence of L3 (infectious) stages and that of earlier (non-infectious) developmental stages. This distinction acknowledges the fact that not all mosquitoes susceptible to the parasite are actually suitable hosts for its complete development. However, even these studies have their limitations. For example, before the L3 stage, filariids infecting mosquitoes cannot be confidently identified to species using morphological traits, although PCR techniques can now be used for species-level identification (Licitra et al. 2010, Scoles and Kambhampati 1995). In addition, mosquitoes found with infectious L3 stage *D. immitis* may not encounter a heartworm-susceptible host to feed on.

Two reviews of mosquito heartworm vectors in the United States have been conducted in the past several decades (Bowman and Atkins 2009, Scoles et al. 1998). Information from these and more recent published studies is summarized in Table 1. In our presentation of naturally infected mosquito species

with L3 filariids presumed to be *D. immitis*, we include collection states and year to provide a sense of geographic distributions of vectors. However, these reports are limited by their sporadic and geographically focal nature. Consequently, we cannot conclude that states excluded from Table 1 lack vector competent mosquito species or have no cases of dog heartworm. A wide-scale organized survey at the state or regional level would be most informative to accurately describe the role of vectors in heartworm epidemiology in the US, but this has never been undertaken.

In Table 2, we propose key criteria, ranked in order, which should be used for identifying the most important mosquito vectors of dog heartworm in the United States. These criteria include the detection of L3 *D. immitis* in wild-caught mosquitoes, vector distribution overlapping with areas of high heartworm prevalence or incidence in domestic animals and/or wildlife, observed propensity for feeding on heartworm-susceptible hosts in nature, observed propensity for high feeding frequency on microfilaremic reservoir host species in nature, and confirmation of vector competence in the laboratory. The strongest evidence that a mosquito species is indeed an important heartworm vector in a region is the detection of infective-stage *D. immitis* in wild-caught specimens. This finding demonstrates that the natural biology and behavior of the mosquito actually do allow it to feed on microfilaremic hosts, become infected, and transmit infectious heartworm to another vertebrate host. If the mosquito species is found in areas of high heartworm prevalence, then it is likely to encounter microfilaremic hosts and be infected with microfilariae at a higher rate. The mosquitoes being considered must also be known to feed on microfilaremic and susceptible vertebrate hosts in which *D. immitis* can complete its life cycle. Vector competence of mosquito species can be investigated by monitoring and quantifying the development of *D. immitis* microfilariae to infective stage in experimentally infected mosquitoes, and this technique is often the first step towards implicating a potential vector. Wild-caught mosquitoes may also be used in experimental infections, as this kind of assay at least provides information specific to the suitability of local mosquito strains. It is helpful to

note that many of the criteria nearer the top of the list can be inferred if other criteria are met. If a wild-caught mosquito is found to be infected with L2 stage *D. immitis*, then it can be assumed that it does feed on microfilaremic hosts in the wild and does feed on animals in which heartworm infections become patent. The same principle applies to finding a mosquito with L3 stages, and this carries the added assurance that *D. immitis* can develop fully in the local strain of that mosquito species.

We have identified top dog heartworm vector candidates from those species that are found naturally infected with L3 *D. immitis and* are abundant in states where dog heartworm incidence was high in 2010. High heartworm incidence areas were defined as those with 50 or more new cases per reporting clinic over the year of 2010 (www.heartwormsociety.org). To aid veterinarians' recommendations to their clients, we have provided the preferred habitats and time of peak feeding activity for these mosquito species in Table 3.

Factors affecting vector competence for dog heartworm Here, vector competence refers to the ability of mosquito hosts to be infected by *D. immitis* microfilariae and to harbor them through L3 stage development; whereas refractoriness indicates the degree to which parasite development terminates before the L3 stage is reached. Interspecies and intraspecies variation in mosquito vector competence for *D. immitis* has partly been attributed to corresponding differences in physiologic and anatomic mechanisms of defense and refractoriness to infection (Beerntsen et al. 2000, Russell and Geary 1996, McGreevy et al. 1978, Michalski et al. 2010, Nayar et al. 1988). The vector efficiency index (VEI) was developed by Kartman in 1954 as a metric to compare vector competence across species and strains of mosquito, and it is still widely used today (Russell and Geary 1996, Tiawsirisup 2007, Tiawsirisup and Nithiuthai 2006, Lai et al. 2000). VEI is calculated by dividing the mean number of L3 stage parasites found in mosquitoes 15 days after infection by the mean number of microfilariae ingested (Kartman 1954). Engorged female mosquitoes are dissected immediately after blood feeding to obtain an

estimate of microfilariae ingested. Estimating VEI is more meaningful than either the number of microfilariae or L3 stage parasites alone, because mosquitoes with highly effective anti-parasite defense mechanisms may have high microfilariae numbers initially, but low numbers of surviving L3 stages. As a consequence, VEI is a more accurate measure of mosquito vector competence for *D. immitis* transmission than vector competence alone.

Vector competence and VEI can be influenced by several factors including the size and shape of the cibarial armature in the mosquito foregut; anticoagulant activity of mosquito salivary proteins on the bolus containing microfilariae; mosquito immunological responses to all stages of the filariid; and genetic differences which can determine the presence and degree to which these and other mosquito defenses act across species and genetic strains of mosquitoes. Some mosquito species possess a well-developed cibarial armature, which is a comblike structure in the mosquito foregut that can mechanically damage microfilariae as they are pumped through the foregut in the ingested bloodmeal. This damage can significantly reducing the number of viable parasites reaching the mosquito midgut (McGreevy et al. 1978, Coluzzi and Trabucchi 1968). Studies designed to determine the effect of the cibarial armature on filarial survival administered a related parasite (*D. repens)* via enema into the mosquito midgut and found that bypassing the cibarial armature increased numbers of developing larvae in the mosquito (Coluzzi and Trabucchi 1968). This remained true even when microfilariae were introduced by this technique in mosquito species that had previously been resistant to *D. repens* infection. These observations suggest that cibarial armature structure may be one factor of the resistance of some mosquitoes to infection with *D. immitis*, although, because of the specificity of armature and filariid interactions, further studies are needed to determine which species combat heartworm infection in this manner (McGreevy et al. 1978).

Differences in the number of viable microfilariae in the midgut after bloodfeeding have been observed in *Culex* species as compared to *Anopheles* or *Aedes* mosquito species fed on blood of the same microfilarial density (Kartman 1953, Tiawsirisup and Nithiuthai 2006, Hu 1931, Russell and Geary 1992). Although the effectiveness of cibarial armatures among these genera could be partly responsible, the extent of anticoagulant activity in the mosquito has also been implicated as a factor modulating vector competence between these genera (Kartman 1953; Russell and Geary 1992, 1996; Lowrie 1991; Nayar and Sauerman 1975). Clotting of the blood bolus in the mosquito midgut can reduce the severity of the microfilarial infection in several ways. Physical impediment to migration may prevent microfilariae from reaching the mosquito hindgut and Malpighian tubules for continued development and oxyhemoglobin crystals formed as part of the clotting process may themselves cause mechanical damage to microfilariae in the midgut. These actions entrap and kill the microfilariae, allowing the mosquito to excrete and digest them before being infected (Kartman 1953, Beerntsen et al. 2000, Michalski et al. 2010, Russell and Geary 1992, Lowrie 1991, Nayar and Sauerman 1975).

Because filarial worms are too large for phagocytosis, the primary mosquito immune responses to filarial infection are intracellular melanization and hemocyte-mediated melanotic encapsulation of larvae that survive or escape mechanical defenses of the midgut (Grieve et al. 1983, Christensen 1981). The microfilarial stage of *D. immitis* is most likely to be melanized and encapsulated than L2 and L3 stages (Tiawsirisup and Nithiuthai 2006, Christensen 1981, Phillips 1939). Larvae sequestered within the digestive tract can then be excreted from the mosquito, while those encapsulated in the hemocoel remain immobilized and die. Given the genetic basis of mosquito defensive interactions, the susceptibility of laboratory-raised mosquitoes may not be representative of all natural populations (Kartman 1953, Beerntsen et al. 2000, Tiawsirisup and Nithiuthai 2006, Theis et al. 2000). In fact, different susceptibilities to heartworm infection have been observed among different geographical strains of the same species of mosquito as well as different laboratory strains. In 2007, Tiawsirisup found

the VEI of Thai strain *Ae. albopictus* ranged from 2.4 to 20 when exposed to a range of microfilarial doses (633-5000 microfilariae/mL), whereas groups of a US strain *Ae. albopictus* given the same doses exhibited a VEI range of 4.2 to 58.3. Also, in 1990, Bradley et al. were able to produce susceptible and refractory substrains of *Ae. aegypti* after three generations of selection from the same parental stock, and these substrains have continued to breed true since their isolation (Nayar and Knight 2002). It is also plausible that strain-strain interactions between *D. immitis* and its mosquito vectors could determine the outcome of host-parasite interactions during infection.

Important factors affecting mosquito species importance for transmission The relative role of competent mosquito vectors in the ecology and epidemiology of heartworm can be influenced by susceptible host and microfilaremic reservoir encounter rates, circadian and seasonal cycles of mosquito activity, seasonality of heartworm development in the mosquito vector population, periodicity of circulating microfilariae in vertebrate hosts, the source and frequency of the vector's blood meals, and the interaction of host and mosquito habitats. This is not a comprehensive list and other important factors are likely.

Although the vector efficiency index is a useful measure to compare vector capacity across mosquito species (Kartman 1954), those with low VEI values could still be major sources of dog heartworm infection. This may be true if less competent vectors are sufficiently exposed to both infected and susceptible hosts. A mosquito's chance of first encountering an infected host and then a susceptible host is driven by host preference; vector and host abundance; host defensive behavior, vector feeding behavior; mortality rates for parasite-infected mosquitoes; and regional patterns of activity and availability of hosts, including domestic animal and wildlife reservoirs of infection (Grieve et al. 1983, Russell and Geary 1996, Vezzani and Carbajo 2006).

Because many different putative vectors of *D. immitis* could be present in the same region, it may be helpful to evaluate the likelihood of heartworm transmission in relation to seasonal and circadian cycles of mosquito activity (Bowman and Atkins 2009, Knight and Lok 1998, Lok et al. 1998). Periods of peak mosquito activity tend to fall into diurnal (day feeding), crepuscular (dawn/dusk feeding), and nocturnal categories. Of demonstrated US vectors, the most commonly reported species are *Ae. vexans, Anopheles punctipennis,* and *Ae. trivittatus*, all of which have crepuscular biting activity, whereas the invasive *Ae. albopictus* is an aggressive diurnal feeder. This information may be useful in determining timing of animal exposure risk with possible vectors. If the prevalent vector species in an area are predominantly diurnal or crepuscular, then the use of pet-safe mosquito repellant and the restriction of domestic animal access to the outdoors during times of peak mosquito activity could be effective (Miller and Crosbie 2011).

As with many filarial infections, the peak microfilarial density of *D. immitis* in a host's peripheral circulation occurs during the late afternoon or evening, presumably synchronizing with circadian cycles of key vector activity (Kartman 1953). Diurnal *Aedes* spp. feeding during times of lower microfilarial density may benefit from lower parasite burden. In contrast, the feeding behavior of nocturnal *Culex* spp. could increase the likelihood of successful development of *D. immitis* by ensuring that high enough doses of microfilariae are ingested to overcome the mosquitoes' mechanical and physiological defenses. In addition, different geographic strains of *D. immitis* could have different periodicities that can increase transmission in light of the biological and ecological characteristics specific to locally important vectors (Bowman and Atkins 2009, Grieve et al. 1983).

Predictive models based on isolines of accumulated HDUs have confirmed the seasonality of heartworm prevalence across all but the most southern regions of the United States (Knight and Lok 1998, Lok et al. 1998). In most areas, the development of *D. immitis* in mosquito vectors is halted during

the winter months by several days with temperatures below the threshold necessary for continued filarial development. Although infective heartworm larvae have been recovered from overwintering mosquitoes, they were assumed to be an unlikely source of heartworm infection during winter months due to the inactivity of mosquitoes in winter diapause (Knight and Lok 1998). Some have reported a disruption of the mosquito activity as a result of heartworm larval development (Berry et al. 1987, 1988), raising the possibility that infected mosquitoes may not exhibit normal overwintering patterns for their species. Additional studies on the effects of *D. immitis* infection on the overwintering behavior of mosquito vectors are needed to fully understand this effect.

The source and average number of blood meals taken by local vectors of *D. immitis* will also determine the efficiency with which heartworm can spread in that ecosystem(Grieve et al. 1983). If a generalist mosquito species is the most abundant or most competent heartworm vector in an area, then *D. immitis* would spread more efficiently if the available hosts for the mosquito vector are mammals within which *D. immitis* can establish a microfilaremic infection. This would ensure continued propagation of the heartworm life cycle, as opposed to its termination in amicrofilaremic or dead-end hosts such as humans, cats, or birds. If, however, the most abundant or competent mosquito vector in an area is a mammal specialist, then the limiting factor for the local spread of heartworm may be vector longevity and multiple feeding rate on typical hosts for *D. immitis* (Grieve et al. 1983, Day 2005, Cancrini et al. 2006). In reality, it is more likely that a few mosquito species will be the predominant vectors in an area, and that these would cover a range of host preferences, activity patterns, and habitats, which should be analyzed to determine their relative contributions to heartworm transmission dynamics in that system (Cancrini et al. 2007, Cancrini 2003).

When describing the epidemiology of dog heartworm, it is essential to take into account the local vector preferences for breeding sites and feeding locations (if known), because these characteristics will

drive the spatial and temporal patterns with which vectors encounter microfilaremic hosts. In a study by Sacks et al. (2004), an accurate predictive model for the distribution of heartworm among coyotes in a sylvatic environment was based on their proximity to suitable areas for the breeding and host-seeking preferences of the predominant mosquito vector, *Ae. sierrensis*. The predictive accuracy of the model was improved by the inclusion of accumulated average temperatures to account for the HDUs required for the development of *D. immitis* in the mosquito vector. Data regarding recent rainfall in the area were used to partially account for the subsequent effects that droughts or increased rainfall can have on moisture, humidity, and the availability of breeding sites necessary for the maintained longevity and presence of mosquitoes during the transmission season (Bowman and Atkins 2009, Sacks et al. 2004, Miller and Crosbie 2011).

Heartworm transmission is also likely to be dependent on interactions between habitat type and the propensity of local vectors to feed in those habitats. In a suburban or urban area, mosquitoes that are peridomestic or endophagic (preferring to feed in areas surrounding human dwellings or within human dwellings, respectively) will more likely play a more important role in heartworm transmission to domestic pets than vectors preferring forested (sylvatic) habitats (Grieve et al. 1983). Sylvatic mosquitoes are more likely to be important in rural areas and areas where pets are outdoors during times of peak mosquito activity (Miller and Crosbie 2011). The presence of stray dogs and/or wild canids such as coyotes or foxes may also play a role in maintaining the sylvatic cycle of heartworm independent of the availability of domestic dog hosts.

Temperature and climate change Shifting weather patterns and climate change have been implicated as possible factors facilitating increases in prevalence of *D. immitis*, partly because of range expansion of vectors and faster parasite development with increasing temperatures (Genchi et al. 2002, Otranto et al. 2009). However, impacts of climate change are likely complex and may contribute to

decreases in transmission under some instances (Harvell et al. 2009). Another short-term effect of climate change may be in the form of increased mosquito population densities after prolonged periods of rainfall, which have been shown to be predictive of increased heartworm prevalence (Sacks et al. 2004), although excessive rainfall may flush mosquito larvae from their habitat (Koenraadt and Harrington 2008). Climate change can also lead to the introduction and spread of competent invasive mosquito species such as *Ae. albopictus*, which has been suggested to be important in the changing distribution patterns of heartworm and its introduction into new habitats (Cancrini et al. 2007, Otranto et al. 2009, Cancrini et al. 2003). In Italy, the rapid spread of *Ae. albopictus* and laboratory confirmation of higher *D. immitis* infection rates as compared with native *Cx. pipiens* has raised concerns over its role in newly developing foci of heartworm infection over the last decade in previously nonendemic areas (Cancrini et al. 2007, Otranto et al. 2009, Cancrini et al. 2003).

Conclusion Recently, failures of standard preventative antihelminthic treatments against *D. immitis* infection in dogs, including ivermectin-based prophylactic drugs, have been reported in the United States, especially in areas of high heartworm challenge (Bourguinat et al. 2011a,b; Blagburn et al. 2011, Hampshire 2005). This challenge highlights the importance of understanding the ecology of mosquito vectors of dog heartworm and *D. immitis* epidemiology. Given their critical link in the heartworm transmission cycle, mosquito vectors should be targeted in disease management programs (Bowman and Atkins 2009). Treatment of heartworm disease in vertebrate hosts can only affect the heartworm development cycle between the L3 stage larva and the production of microfilariae. Vector control measures reduce the incidence of heartworm by lowering the population of infective mosquitoes. A multifaceted approach to disease management that includes parasite and vector control would be less selective for drug resistance in the parasite population. Vector management is also an attractive option for areas in which wildlife reservoirs of *D. immitis* would continue to expose the mosquito population to microfilariae. This approach could be preferable if the alternatives are culling or treating infected

wildlife, which is often costly, controversial, or ineffective unless conducted on a large comprehensive scale.

Because dog heartworm can potentially be transmitted by a wide range of mosquito species, vector control must be adapted for the specific geographic location and vectors of interest. The current vector status of many natural populations of mosquitoes remains unknown; however, several demonstrated vectors are abundant and have a wide distribution across the country (Bowman and Atkins 2009, Scoles et al. 1998, Watts et al. 2001). Depending on the key vector species ecology, larval source reduction may be an effective means of reducing heartworm vector populations. Veterinarians can also ensure that their clients are aware of the risk that mosquito vectors pose to humans (although rare) and their pets. Recommendations should include the use of insect repellant for humans and pets, window and door screens, and avoidance of peak mosquito activity times in the area. We suggest Table 3 as a starting point for veterinarians looking for information regarding the habitat and feeding activity of mosquito vectors of heartworm on which to base their recommendations to clients.

Table 1

Mosquito species collected naturally infected with L3 filariids (i.e. indicative of vector competence) presumed to be *Dirofilaria immitis* in the United States

Species	State[x]	Year of collection
*Ae. albopictus**	FL, GA, LA	1994[1], 1998[2], 2006, 2007[3]
*Ae. canadensis**	CT, FL, MA, NJ	1974-1977[4–6], 1996[7]
Ae. cantator	NJ	1974[4]
Ae. excrucians	CT, MA	1939[8], 1976, 1977[5,6]
Ae. infirmatus	FL	1997[7]
Ae. sierrensis	CA	1996[9]
Ae. sollicitans	CT, NC, NJ	1974[4], 1976, 1977[6], 1985-1987[10,11]
Ae. sticticus	AL, MA	1975-1978[5,12], 1986[13]
Ae. stimulans	CT	1976, 1977[6]
Ae. stimulans group	MA	1975, 1976[5]
Ae. taeniorhynchus	FL, NC	1985-1987[10,11,14]
Ae. triseriatus	IN	1993-1995[15]
*Ae. trivittatus**	AL, IA, IN, OK, TN	1975[16], 1982[17], 1985[18], 1986[13], 1988[19], 1993-1995[15]
*Ae. vexans**	AL, CA, CT, FL, IN, LA, MD, MI, MN, NJ, NY, OK	1963, 1964[20], 1974-1978[4,6,12,21–23], 1982[17,24,25], 1986[13], 1988[19], 1995-1997[9,15]
An. bradleyi	NC	1985, 1986[10,11]

An. crucians	AL, FL, GA	1986[13], 1996, 1997[7], 2006, 2007[3]
An. freeborni	CA	1996[9]
*An. punctipennis**	AL, GA, IA, KY, MA, MD	1939[8], 1975-1978[12,16,22], 1982[24], 1983[26], 1986[13], 2006, 2007[3]
*An. quadrimaculatus**	LA, MA, MD, MI, NY	1939[8], 1964[27], 1974, 1975[21], 1977[23], 1993-1996[7,15]
Cx. nigripalpus	FL	1983[14], 1996, 1997[7]
Cx. p. pipiens	MI	1974, 1975[21]
*Cx p. quinquefasciatus**	AL, FL, LA	1967, 1968[28], 1982[24], 1983[14]
Cx. salinarius	MD, NC, NJ	1974[4], 1976[22], 1986[10]
Ps. columbiae	LA	1996[7]
Ps. ferox	CT, FL	1976, 1977[6], 1996, 1997[7]

[x]This column represents the states in which mosquitoes naturally infected with *D. immitis* of any stage were collected. There have not been thorough surveys conducted in every state, so states not indicated here should not be considered free of *D. immitis* or its vectors. *Species addressed in further depth in Table 3 due to their proposed importance as vectors of heartworm across the US.
Footnoted references can be found in Appendix E.

Table 2

Key criteria for identification of dog heartworm vectors (Listed in order of importance)

- Detection of wild-caught mosquitoes infected with L3 stage *D. immitis*
- Vector distribution overlaps with areas of high heartworm prevalence or incidence in wildlife or domestic animals
- Feeding on susceptible vertebrate hosts in nature
- High feeding frequency on microfilaremic vertebrate reservoirs in nature
- Demonstrated vector competence of field strains (susceptibility to infection and demonstrated development of L3 larvae) in laboratory studies

Table 3

Geographic range, feeding behavior, and habitat information of important mosquito vectors of *Dirofilaria immitis* found across the United States(Means 1987)**.**

Species	No. of high incidence states[α]	Total no. of states[β]	Daily peak of feeding activity	Habitat notes
Ae. albopictus	3	3	Diurnal(Gratz 2004)	Cosmopolitan (found across many different habitats), container breeding.
Ae. canadensis	1	4	Crepuscular, diurnal	Sylvatic, swampland. Breeds in clean, cool water in woodland pools, also open grassy roadside ditches or dark cellars.
Ae. trivittatus	3	5	Early evening	Sylvatic, swampland. Not found in densely forested areas. Breeds in temporary woodland pools with substantial vegetation.
Ae. vexans	4	12	Early evening to midnight	Endophagic. Can migrate long distances toward urban areas and lights. Breeds in floodwater areas with temporary pools, flooded pastures and partially shaded woodland pools lined with decaying vegetation.

An. crucians	3	3	Dusk and nocturnal	Sheltered areas, does not enter houses. Found in barns, pastures, stables, culverts, and under houses. Breeds in semi-permanent and permanent bogs, ponds, and bayous containing acidic water.
An. punctipennis	3	6	Early evening	Exophagic . Breed in small permanent streams especially when shaded, blocked, or containing emergent vegetation; swamps; small ponds; and containers such as wooden tubs or barrels.
An. quadrimaculatus	1	5	Nocturnal	Endophagic and exophagic. Breeds in swamps, roadside ditches, slow moving streams, canals, lakes, ponds, and even puddles with emergent vegetation.
Cx. quinquefasciatus	3	3	Nocturnal	Breeds in containers, catch basins, storm and waste water effluent

[α]Counts represent states with areas of high incidence of heartworm (>50 cases/reporting clinic in the year 2010) (www.heartwormsociety.org) in which mosquitoes have previously been found with naturally-acquired heartworm infection. [β]Counts represent states in which mosquitoes have previously been found with naturally-acquired heartworm infection.

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CHAPTER TWO

DNA barcoding analysis of wild-caught mosquitoes: quality control analysis of detection threshold, amplification bias, and multiple hosts

ABSTRACT

Mosquito collections were conducted from May 30-October 4, 2012 in Ithaca, NY using a combination of CDC traps, resting boxes, and large vegetation aspirators. Collections were identified to species and blood meal analysis was performed on engorged mosquitoes. Large vegetation aspirators were most efficient for collecting blood fed mosquitoes (2.99/collection-hour), and resting boxes were second (1.04/trap-night). CDC traps collected no blood fed mosquitoes. Quality control analyses were performed on positive controls of local wildlife blood samples to assess detection threshold, success rate, bias, and ability of our DNA barcoding method to identify different ratios of mixed blood. My methods demonstrate that primer bias can consistently favor amplification and identification of one species template over another, and that two hosts within one blood meal can sometimes be matched to database entries with a single query, as was true for two wild-caught specimens. I offer recommendations for blood meal analysis projects depending on availability of resources and research purpose. White-tailed deer were the most abundant identified blood meal host (n=97), and humans were second (n=12). I conclude that *Culiseta melanura* feeding patterns between 13 different species of mammals, birds, and amphibians/reptiles could make it a bridge vector of introduced arboviral disease such as Eastern Equine encephalitis.

INTRODUCTION

Effective control of vector-borne diseases requires confirmation of vector associations with the infectious agent and its hosts. Accurate vector incrimination must be based on the following proofs presented in order of increasing importance: association of vector activity or abundance with disease incidence, vector competence for infection and transmission, and confirmed vector-pathogen-host contact in natural populations (Ledesma and Harrington 2011). Blood meal analysis is a powerful tool for investigating natural vector-host contact patterns by analyzing host origin of blood in wild-caught fed mosquitoes. DNA barcoding is a method of blood meal host determination that distinguishes host species based on polymorphisms in the *cytochrome oxidase I* (COI) gene. DNA extracted from blood fed mosquito abdomens was amplified with degenerate primers and sequenced for matching with entries in The Barcode of Life Data Systems (BOLD)(http://www.boldsystems.org) and NCBI BLAST (http://blast.ncbi.nlm.nih.gov). However, issues persist with processing and reporting database match information and failure rates (Townzen et al. 2008). Amplification and sequencing are limited by DNA recovery from digestion in mosquito midguts, and database inquiries depend on a wide species representation and good quality sequence entries. If these parameters are satisfied, a primer set must be designed or chosen to target vertebrate COI gene sequences without amplifying mosquito COI. Primer sets in the literature have been designed and tested with host species available to researchers in their own study regions (Townzen et al. 2008, Alcaide et al. 2009, Thiemann et al. 2012). Care must be taken to avoid bias in DNA amplification, especially if there is little knowledge of host species present in the collection area. Polymorphisms in target COI sequence between study populations could contribute to amplification bias or mismatch with global DNA barcode databases or barcoding primers

designed for non-local host sequences. Unfortunately, this is rarely reported in the literature. Matching COI sequences to database entries presents the problem of defining a positive match threshold that excludes low quality and unreliable match information. There is little information about the expected match percentage for these comparisons, and these match rates likely differ between primer sets, sample processing, and laboratories.

Nevertheless, knowledge of feeding patterns of putative vector species can provide valuable information for model development and defining risk factors in the case of disease introduction (Brown et al. 2012, Ruiz-Moreno et al. 2012). New York State is home to at least 59 mosquito species (Darsie and Ward 2005), and mosquito-borne pathogens in New York State include West Nile virus, Eastern Equine Encephalitis virus, and dog heartworm (*D. immitis*) (www.diseasemaps.usgs.gov). Many vectors of both human and animal disease are represented in study sites in Tompkins County, NY including *Aedes canadensis, Ae.vexans, Anopheles quadrimaculatus, An. punctipennis, Culex pipiens,* and *Cx. restuans.* These species are present in large numbers in collections from areas with high human and wildlife activity, and mosquito species variation in feeding behavior determines their potential contribution to pathogen transmission between reservoir hosts and humans and domestic animals (Darsie and Ward 2005, Kilpatrick et al. 2005).

Cx. *pipiens* has been studied extensively for its role in West Nile virus transmission and *Cx*. *restuans*, a related and often sympatric species, is assumed to have a similar ecology. Morphological methods of distinguishing adult *Cx. restuans* from *Cx. pipiens* are inadequate when verified against molecular and larval traits (Harrington and Poulson 2008), which

complicates studying these species separately. Studies reporting adult feeding pattern and ecological data for *Cx*. *pipiens* or *Cx*. *restuans* adults in areas where their ranges overlap must use molecular or larval methods of identification, or must acknowledge the limitations of morphological identification; however, many do not. Since blood fed *Cx*. *restuans/pipiens/salinarius* were often caught during my pilot studies (data not shown), I used molecular identification of these species in conjunction with blood meal analyses to determine if they exhibit distinct feeding patterns.

My goal was to understand blood feeding patterns and heartworm infection status of putative mosquito vectors in sites across Tompkins County as an entry point for addressing challenges and optimizing mosquito blood meal analysis. I collected and identified adult female mosquitoes and used DNA barcoding methods to determine the hosts of blood fed mosquitoes and to identify damaged specimens. Heads and thoraces were pooled and screened for *D*. *immitis*. Feeding patterns, abundance, distribution, and seasonality were described for mosquito collections spanning the summer of 2012. Implications for mosquito-borne disease transmission and vector incrimination are discussed.

Materials and Methods

Study sites Collections in Ithaca, NY in 2012 were conducted at the following sites: Sapsucker Woods Sanctuary (42° 28' 37.5492" N, 76° 26' 59.8626" W); SPCA of Tompkins County (42° 28' 19.596" N, 76° 26' 20.9394" W); Ithaca Farmers' Market (42° 27' 1.4754" N, 76° 30' 36.5394" W),Robert Trent Jones Golf Course (42° 27' 29.16" N, 76° 27' 35.64" W), and pilot

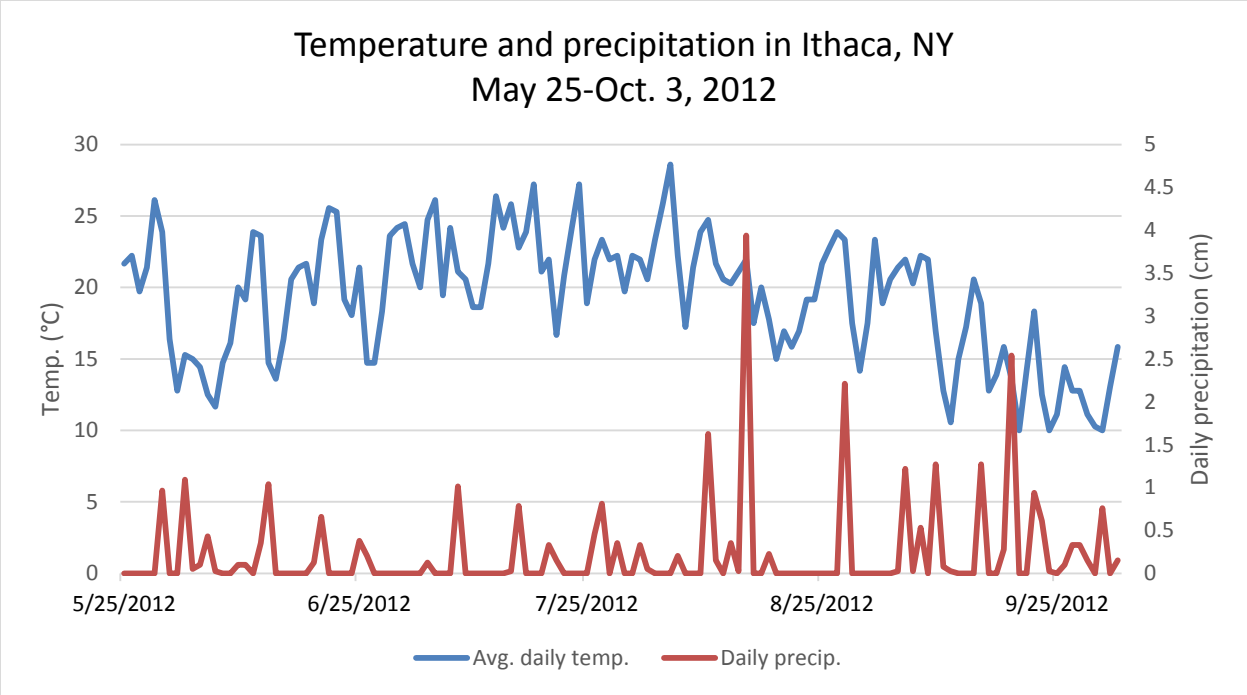

Figure 2.1: Average daily temperature and daily precipitation spanning the collection period (http://www.nrcc.cornell.edu/).

collections were also performed at a residential area (42° 28' 54.9474" N, 76° 24' 33.48" W) and Cass dog park (42° 26' 53.9154" N, 76° 30' 50.8278" W).

Weather station observations of average temperature and daily precipitation in Ithaca over the collection period are reported (Fig. 2.1).

Sapsucker Woods Sanctuary comprises ponds, wetlands, and upland hardwood forest. Collections targeted ground level fern, poison ivy, and leaf litter. The SPCA grounds included a small area of dense shrubs and small trees and an artificial pond with cat tails and other aquatic vegetation. Collections at Robert Trent Jones Golf Course focused on unmaintained areas of brush vegetation such as blackberry, poison ivy, long grass, and fern. The Ithaca Farmers' Market is near a lakeside dock and is bordered by tall grasses, small shrubs, and areas shaded

by hardwood trees. Sites were chosen for their frequent human and animal traffic and for their inclusion or proximity to persistent ponds, woods, containers, and wetlands acting as mosquito breeding habitats.

Mosquito collection and handling Mosquitoes were collected using large vegetation aspirators (Ponlawat & Harrington, 2005), CO_2-baited CDC traps (Service 1995), and resting boxes (Edman et al. 1968). Methods deployed at each site are summarized in Table 2.1.

Each site for vegetation aspiration was divided into 2-4 smaller sampling areas adjacent to cleared paths. Collectors began at specified locations on the path and walked into and along the woods for the duration of the sampling time. Collections were performed 2-3 times per week, and each area within a site was sampled for 10-20 min per collector per visit. Collections were performed between 9:30 and 12:00 hrs based on analysis of previous collection efforts. The collection season spanned May 30-October 4, 2012.

Vegetation aspirators were fitted with 1 gallon mesh paint strainer bags (Master Craft, South El Monte, CA). Sample bags from each 10-20 min collection were immediately placed inside separate sealed plastic bags to prevent mosquito escape. Sample bags were stored in a cooler with ice until returning to the laboratory. Bags were cooled at -20°C for at least 10 min before processing. Contents of each bag were distributed in white plastic trays to facilitate sorting of mosquitoes. Blood fed mosquitoes were placed in individual microcentrifuge tubes to prevent contamination. Non-fed mosquitoes from the same collection bag were pooled by species and placed in tubes. The date and time interval of collection, collection location, collector, total number of female mosquitoes per collection effort, and number of blood fed

female mosquitoes were recorded. These values were also recorded for collection efforts yielding no mosquitoes. All mosquitoes were stored in sample tubes at -20°C or -80°C prior to DNA extraction. Collection, cooling, and sorting of mosquitoes were the same for those gathered from resting boxes and CDC trapping.

Mosquito identification Blood fed female mosquitoes were identified to species by morphological characteristics using a standard morphological key for the study region (Andreadis et al. 2005), except for *Culex restuans, Cx. pipiens,* and *Cx. salinarius*, which were identified using species-specific primers for PCR as described by Harrington and Poulson (2008). These species were identified based on interspecies nucleic acid sequence variation in the first and second internal transcribed spacers (ITS) of the nuclear ribosomal DNA gene array (Crabtree et al. 1995).

DNA extraction DNA extractions for both blood meal identification and *Culex* species identification were performed using a modified PureGene DNA extraction protocol (Gentra Systems, Minneapolis, MN). Heads and thoraces of blood fed *Culex restuans/pipiens/salinarius* mosquitoes were used for *Culex* identification, and abdomens of blood fed mosquitoes were used for blood meal identification. Dissection of abdomens from heads/thoraces was performed with surgical blades sterilized between samples by heating over a flame until glowing red.

Each specimen was homogenized in 300 µl of cell lysis solution and incubated at 65°C for 15 min. Lysate was chilled on ice for 2 min and 100 µl of protein precipitation solution was added. Sample solution was mixed by vortex for 20 secs before being centrifuged at 17900 x g

for 5 min. Supernatant containing DNA was transferred to a tube containing chilled 100% isopropanol to produce a 70% isopropanol final solution. This was inverted multiple times to mix and was held at -20°C for 10 min. Each tube was then centrifuged at 17900 x g for 20 min. Supernatant was poured off and 500 µl of freshly made 70% ethanol solution was added to each tube containing the DNA pellet. Tubes were inverted by hand several times before centrifugation at 17900 x g for 20 min. Supernatant ethanol solution was carefully poured off before an additional 2 min of centrifugation at 17900 x g. Remaining ethanol was removed by pipette, and tubes were left to air-dry for 10 min. 50µl of dH_2O (Gibco) was added before storage at -20°C.

PCR amplification of vertebrate DNA in mosquito blood meals

Optimization of DNA barcoding approach Many protocols and primer sets were evaluated for their success in amplifying vertebrate DNA (cytochrome b or cytochrome oxidase I) from known positive controls and mosquito blood meals for identification with DNA barcoding techniques (Ngo and Kramer 2003, Townzen et al. 2008, Alcaide et al. 2009, Montgomery et al. 2011). Based on preliminary comparisons, I chose primer sets COI long and COI short according to the following DNA barcoding protocol modified from Townzen et al. (2008). Samples that did not amplify well with the COI long primer set were run under the same conditions with the COI short primer set. Samples that still did not amplify after this step were not analyzed further. Attempts at using semi-nested combinations of COI short F and COI long R, as described in Townzen (2008), often resulted in sequences of low match percentage when entered into DNA barcoding nucleotide databases.

DNA barcoding Each 25 µl PCR tube contained 1.5 µl of template solution, 0.5 µl of 10 µM COI long F primer solution, 0.5 µl of 10 µM COI long R primer solution, 2.25 µl of 50 mM $MgCl_2$, 0.5 µl of 10 mM dNTP, 5 µl of 5x GoTaq Flexi buffer (Promega), 0.15 µl of 5 U/µl GoTaq Flexi DNA polymerase (Promega), and 14.6 µl of dH_2O (Gibco). Thermal cycling conditions consisted of 95°C for 5 min, 95°C for 30s, 50°C for 50 s, 72°C for 1 min, and 72°C for 5 min. Cycles 2-4 were repeated an additional 39 times. PCR products were separated on an ethidium bromide-stained 1% agarose gel in 1X TBE buffer for 60 mins at 120V before being visualized and digitally photographed using a Multi Doc-It Digital Imaging System (UVP Inc.).

Each sample was purified and prepared for sequencing as follows: 2 µl of ExoSAP-IT (Affymetrix, Inc.) were added to 5 µl of PCR product and incubated at 37 C for 15 mins and then 80 C for 15 mins. Each sample tube sent to sequencing contained 1 µl of post-ExoSAP-IT PCR product, 1 µl of 10 µM of COI long F primer (COI short F primer was used for COI short primer PCR products), and 16 µl dH_2O (Gibco). Resultant sequences were viewed and edited in CodonCode Aligner (CodonCode Corporation, Dedham, MA) to remove beginning and end sequence that could correspond to primer sequence. Trimmed sequences were entered into the Barcode of Life Database (BOLD, http://v3.boldsystems.org) and NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Accepted positive match values were based on quality control analysis with positive control samples and confirmation of species ranges included in the local area.

All primer sets and amplicon sizes are listed in Table 2.2.

Verification of DNA barcoding technique: quality control, limit of detection, and blood meals of mixed host origin

There are no widely-accepted standards for interpretation of database search results for DNA barcoding of mosquito blood meals. As discussed by Townzen et al. (2008), DNA barcoding identification of mosquito blood meals should incorporate host species availability and distribution in the study area, database matching thresholds, evaluation of chosen primer sets with positive controls, and verified protocols for processing ambiguous results and blood meals of mixed host origin. Mosquito blood meal threshold match percentage and the success/failure rate of sample identification are rarely reported in the literature, and subsequent studies are often left to form their own standards and acceptable rates of failure.

One goal of my study was to verify my methods using positive controls of whole blood collected in heparinized tubes from local wildlife patients of the Janet L. Swanson Wildlife Health Center, an annex of the Cornell University Hospital for Animals. Heparinized blood was stored at -20°C after collection until DNA extraction.

For single host species blood meal assays, DNA was extracted from three µl of whole blood representing average *Ae. aegypti* blood meal volume (Jeffery 1956). Single species extractions were amplified using both Townzen Long and Townzen Short primer sets according to methods described above (Townzen et al. 2008). Positive identification threshold of host origin was determined. Limits of detection were set by analyzing ten- and two-fold serial dilutions of single host blood meal DNA extractions until a match over the established threshold could not be made. Limit of detection was reported in nanograms of template DNA.

Assays of mixed host blood meals were performed in a final volume of 3 µl at 1:1, 1:2, and 2:1 ratios. Two-species reciprocal combinations comprised samples from mammal, bird, amphibian, and reptile species common in Central New York. Special attention was given to mixes of mammal blood with amphibian, reptile, and bird blood to explore sources of identification bias. COI Long and COI Short primer sets were used to amplify all combinations (Townzen et al. 2008).

Sequences were matched against reference entries on BOLD and BLAST.

Table 2.1: Collection methods employed at each site for the 2012 collection season. All collections were performed in the morning.
CDC=CO_2-baited CDC trap
RB=resting box
VC=vegetation aspirator

Sapsucker Woods	Golf Course	Cass dog park	Residential area	TSPCA	Farmers' Market
CDC, RB, VC (AM)	VC(AM)	VC (AM)	VC (AM)	CDC, RB, VC(AM)	CDC, RB, VC(AM)

CDC=CO_2-baited CDC trap
RB=resting box
VC=vegetation aspirator

Table 2.2: Name, sequence, and expected amplicon size of primer targets. Blood meal analysis, mosquito barcoding, *D. immitis* screening, and ***Culex restuans/pipiens/salinarius* identification.**

Primer name	Primer Sequence Forward 5'-3'	Primer Sequence Reverse 5'-3'	Expected Amplicon
COI long[a]	AACCACAAAGACATTGGCAC	AAGAATCAGAATARGTGTTG	663 bp
COI short[a]	GCAGGAACAGGWTGAACCG	AATCAGAAYAGGTGTTGGTATAG	324 bp
LCO1490/HCO2198[b]	GGTCAACAAATCATAAAGATATTGG	TAAACTTCAGGGTGACCAAAAAATCA	~650 bp
DI-COI[c]	AGTGTAGAGGGTCAGCCTGAGTTA	ACAGGCACTGACAATACCAAT	203 bp
CP16[d]	PQ10, R6, S20 multiplex	GCGGGTACCATGCTTAAATTTAGGGGGTA	
PQ10[d]	CCTATGTCCGCGTATACTA	CP16	698 bp
R6[d]	CCAAACACCGGTACCCAA	CP16	506 bp
S20[d]	TGAGAATACATACCACTGCT	CP16	175 bp

[a](Townzen et al. 2008)
[b](Folmer et al. 1994, Cywinska et al. 2006)
[c](Rishniw et al. 2006)
[d](Crabtree et al. 1995)

Results

Mosquito collection The majority of trapping was conducted in Sapsucker Woods and the Ithaca Farmers' market in June, July, and August (Table 2.3); Most collection-hours were accumulated at Sapsucker woods, SPCA, and farmers' market sites in June and July (Table 2.4). Mosquito species yield per collection-hour or trap-night varied between locations and methods (Figs. 2.2-2.5). Resting boxes were the most efficient methods for trapping *An. quadrimacultus* and *An. punctipennis* across all sites (Figs. 2.2-2.4), and they were the second-most efficient collection method targeting blood fed mosquitoes (Fig. 2.7). The most efficient method was vegetation aspiration. CDC traps collected no blood fed mosquitoes (Fig. 2.7), and yields varied the most between locations. *Culiseta melanura* and *Cx*. *restuans/pipiens/salinarius* complex mosquitoes comprised a greater part of CDC collections from the farmers' market and SPCA.

Ae. canadensis, *Ae. triseriatus,* and *Ae. vexans* were the top three most collected mosquitoes per CDC trap night in Sapsucker woods (Figs. 2.2-2.4).

Anopheles punctipennis, *Culiseta melanura*, *Aedes canadensis*, and *An. quadrimaculatus* comprised the majority of my collections, representing 18.5%, 17.3%, 15.5%, and 14.1%, respectively. Collection methods targeting the broadest mosquito species diversity changed with study site. Sapsucker woods vegetation aspirations collected at least 15 mosquito species, but, at the farmers' market, aspiration collection collected only 2 species, and resting box yields were the most diverse with seven different species. SPCA collections were most diverse in CDC traps, which yielded seven species over the collection period (Figs. 2.2-2.4).

Blood meal analysis of wild-caught mosquitoes Host and mosquito species of successfully identified blood meals (n=166) are presented in Tables 2.5-2.7. I obtained the most identifiable blood meals from *An. punctipennis* (24.7%), *An. quadrimaculatus* (21.1%), and *Cs. melanura* (13.3%) out of a total 166 blood meal identifications (Tables 2.5-2.7). Overall, mammal, avian, and amphibian/reptile hosts were identified as 72.3%, 22.3%, and 5.4% of blood meals. Human and white-tailed deer were the most commonly identified hosts. The majority of deer blood meals were taken by *An. punctipennis* and *An. quadrimaculatus*. *Aedes canadensis* was the most common human-fed mosquito in my collections, although most mammal-fed species included human blood meals (Table 2.5). Matches to red deer and moose were most likely white-tailed deer based on my quality control analyses.

Culiseta melanura was the most common avian-fed mosquito and the most generalist in number of host species identified: 2 mammal, 12 avian, and 1 amphibian (Tables 2.5-2.7).

Wood thrushes and northern cardinals were the most common avian blood meal hosts (7 blood meals each).

Gray tree frog was the most common amphibian/reptile blood meal host (7 mosquitoes). Five out of a total nine amphibian/reptile blood meals were taken by *Cx. territans* (Table 2.7).

I believe that a northern cardinal/white-tailed deer multiple blood meal was taken by an *Ae. canadensis*. A multiple blood meal was also identified in a *Cs. melanura* that fed on both an Eastern phoebe and a wood thrush. These are suspected mixed blood meals due to different host identifications when amplified with different primers, which was revealed by my quality control analyses to be one way in which mixed blood meals may be identified.

Table 2.3: Trap-nights for the entire collection season by location, trap, and month.
SSW=Sapsucker Woods, FM=Farmers' market, SPCA= Tompkins County Society for the Prevention of Cruelty to Animals, RB=resting box

Location	Trap	Month	Trap-nights
SSW	CDC	May	1
		June	5
		July	4
		August	4
		September	4
	2x2 RB	June	10
		July	2
	2x3 RB	June	10
		July	11
		August	23
	2x4 RB	June	9
		July	11
		August	6
FM	CDC	August	3
	2x2 RB	August	2
		September	2
		October	1
	2x3 RB	August	16
		September	2
	2x4 RB	August	8
		September	5
		October	1
SPCA	CDC	July	4
		August	1
	2x2 RB	July	7
		August	4
	2x4 RB	August	4

Table 2.4: Collection-hours presented by location and month. GC=Robert Trent Jones Golf Course

	Collection-hours (hh:mm)
SSW	20:00
SPCA	4:30
FM	2:00
GC	0:50
Residential	0:40
Cass park	0:15
June	12:55
July	9:40
August	5:30

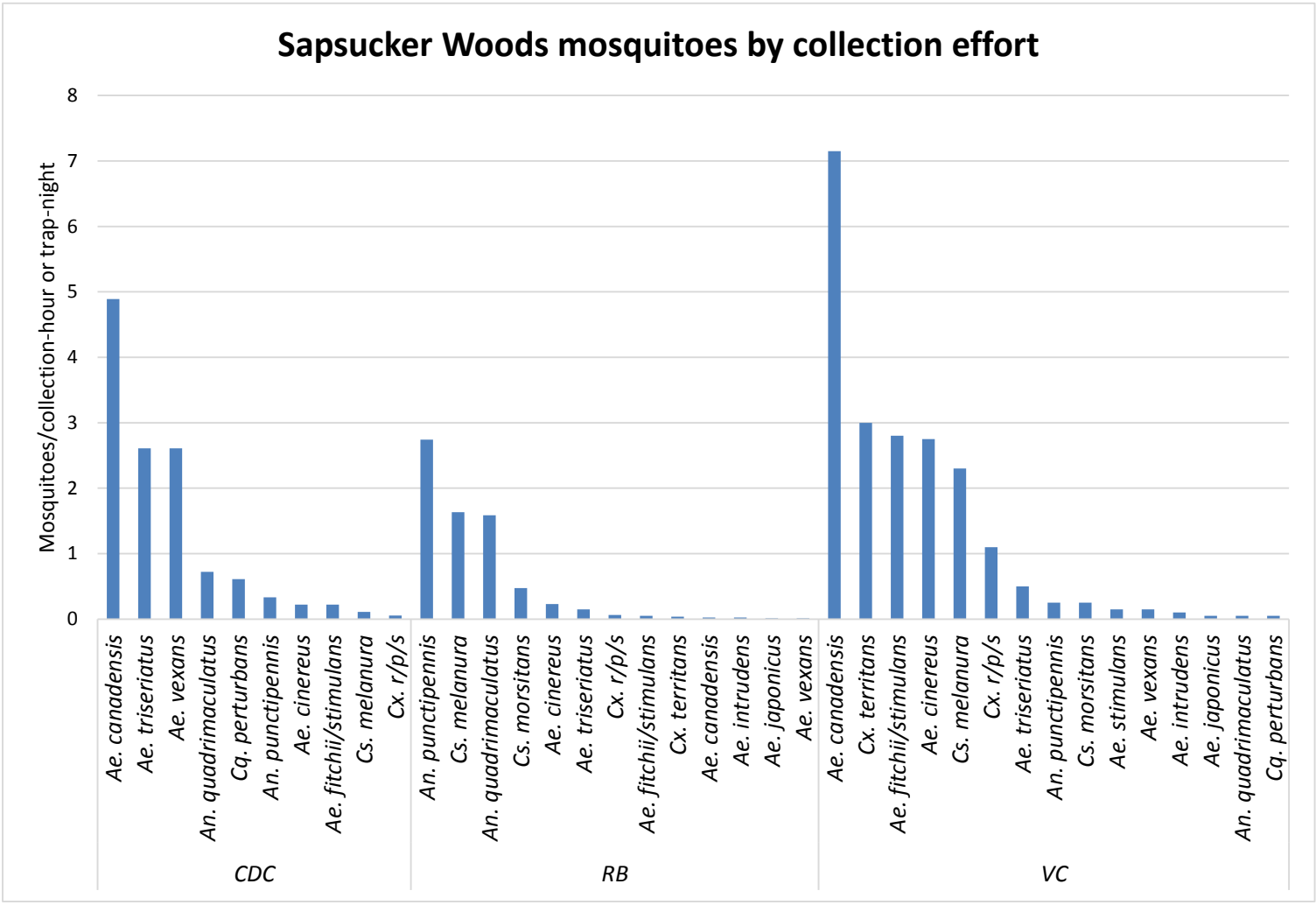

Figure 2.2: Female mosquitoes collected in Sapsucker woods, standardized by trap-nights (CDC, RB) or collection-hours (vegetation collection: VC).

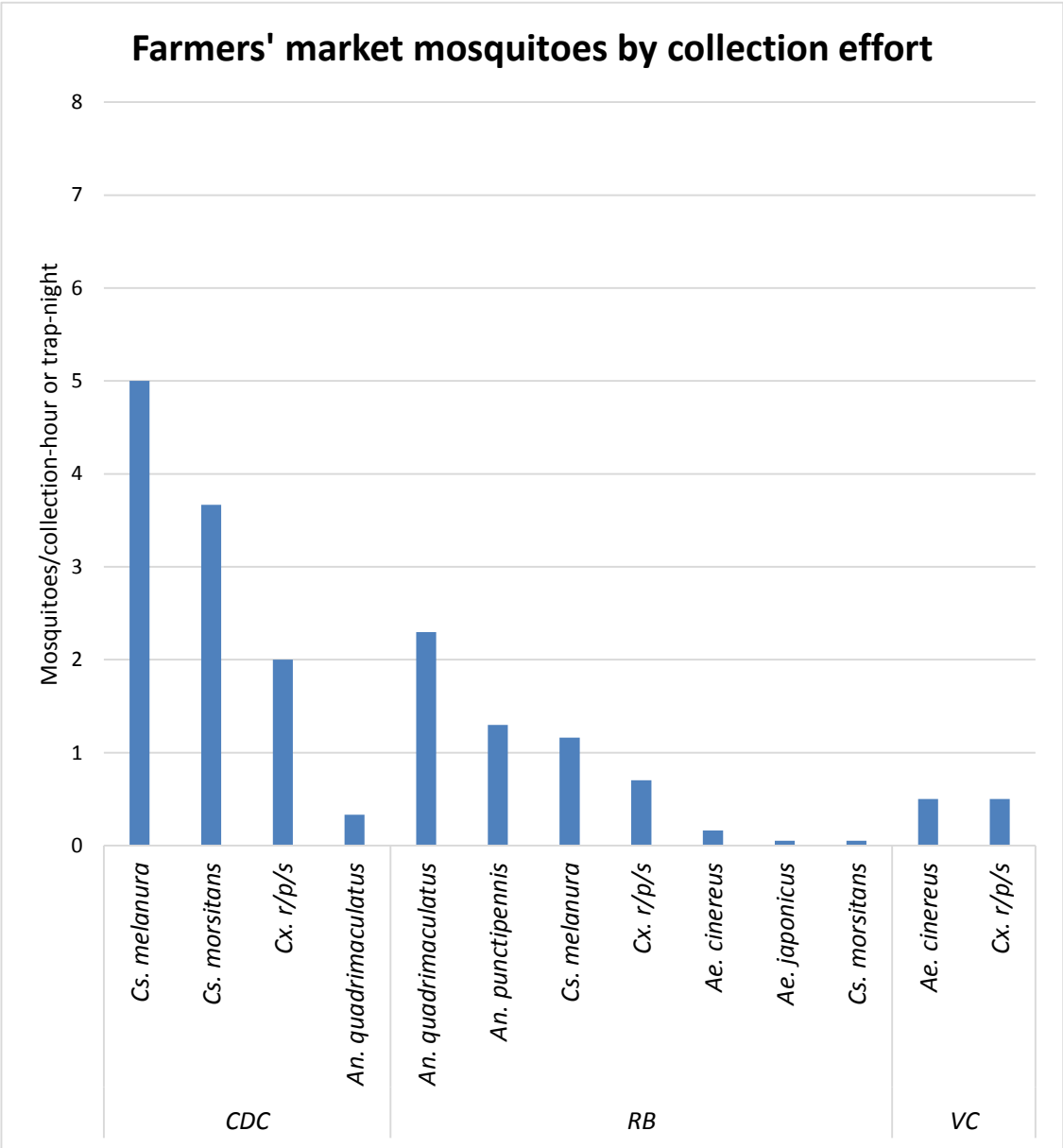

Figure 2.3: Female mosquitoes collected at the farmers' market, standardized by trap-nights (CDC, RB) or collection-hours (VC).

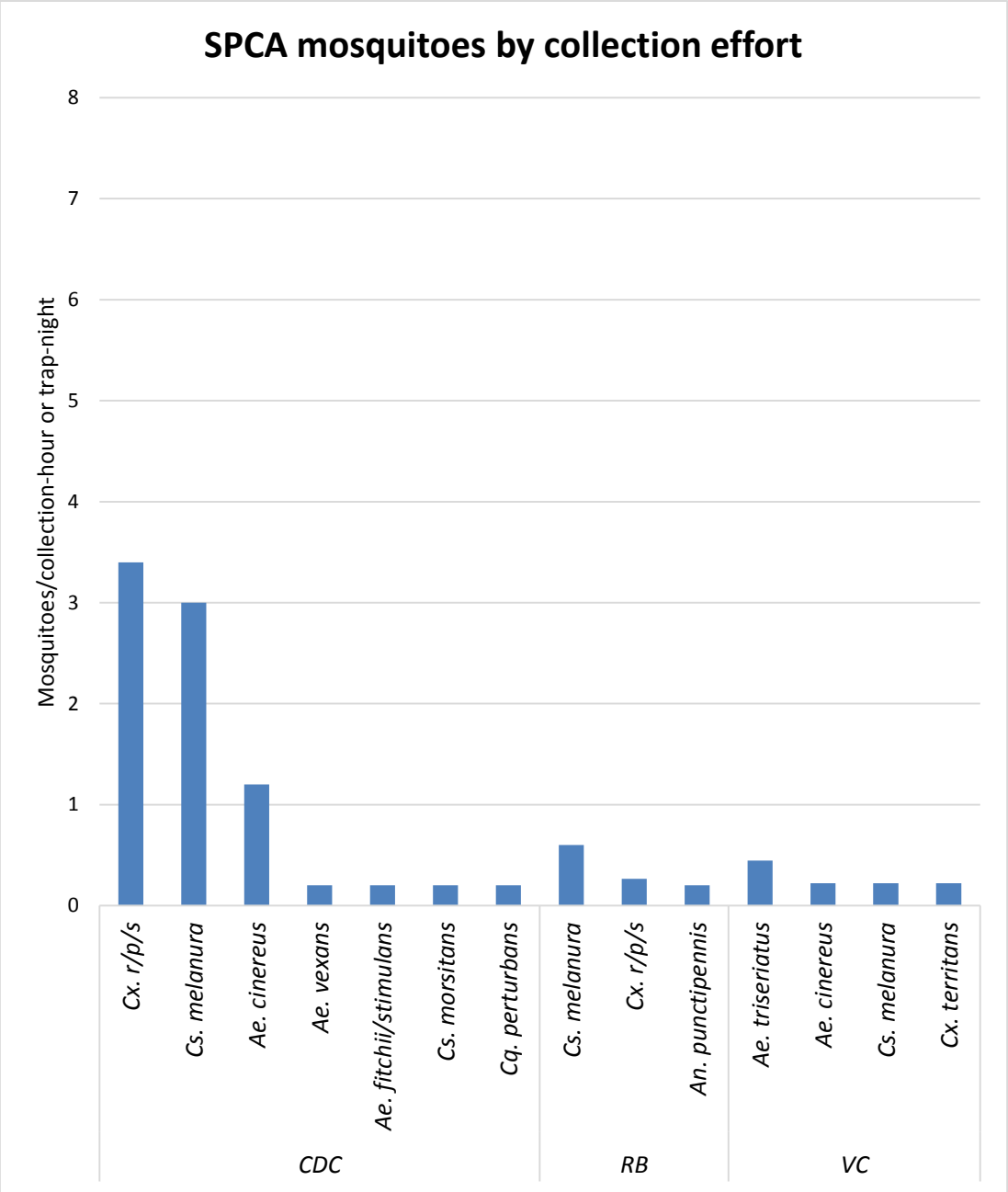

Figure 2.4: Female mosquitoes collected at the SPCA, standardized by trap-nights (CDC, RB) or collection-hours (VC).

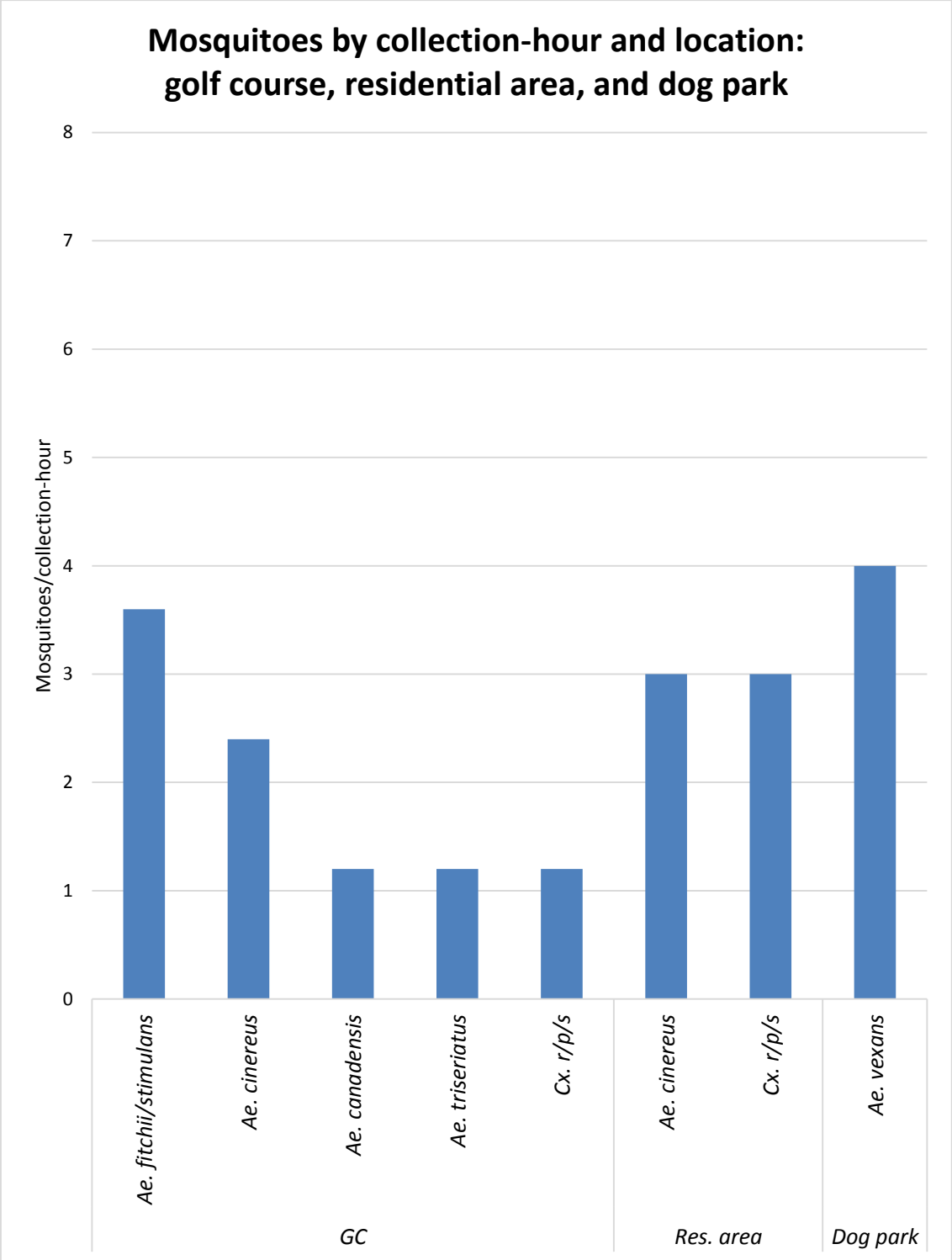

Figure 2.5: Female mosquitoes collected by vegetation aspiration at the golf course, residential area, and dog park, standardized by collection-hours.

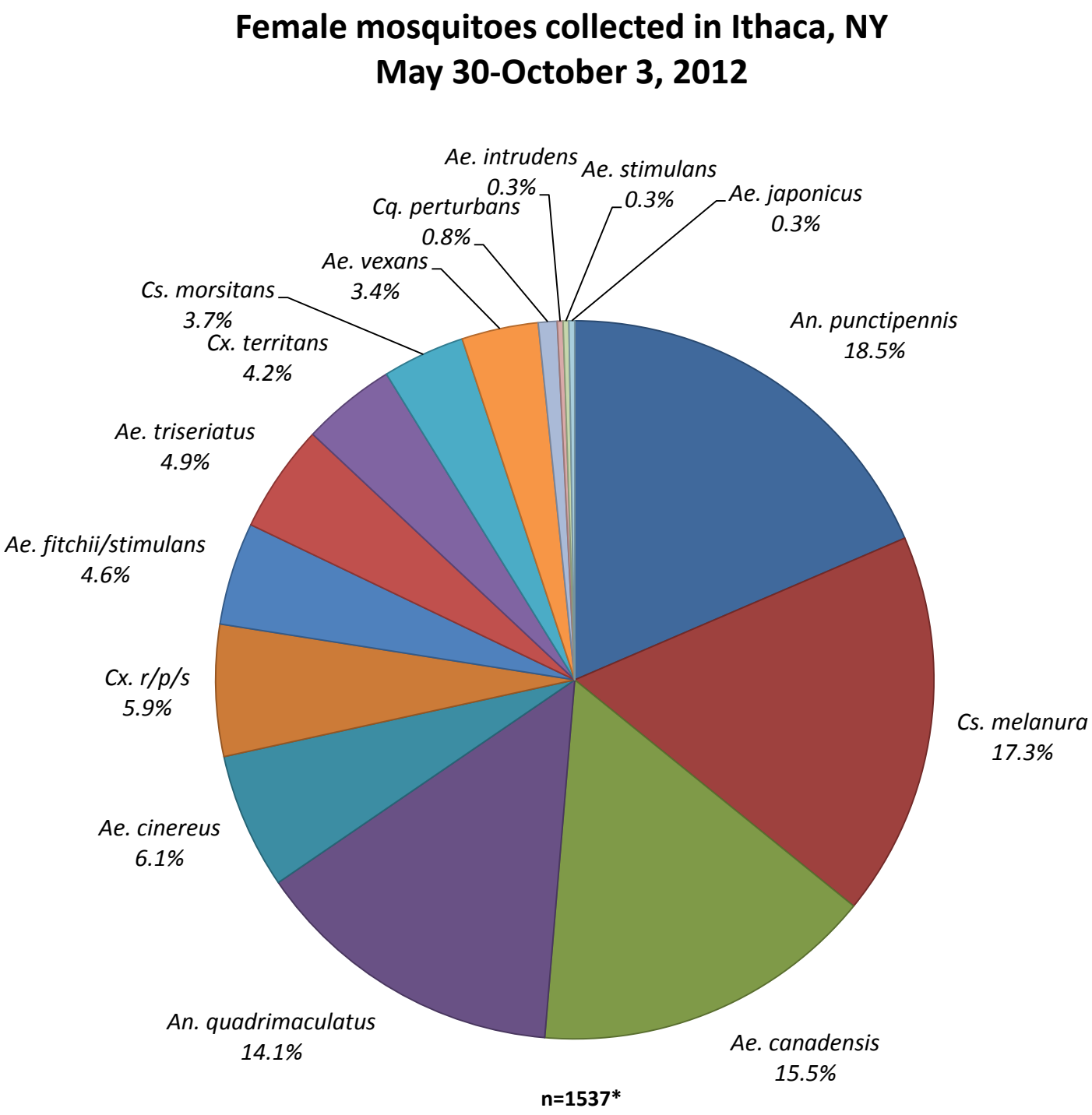

Figure 2.6: Species distribution of identified female mosquito collections. *67 mosquitoes were unidentifiable to species and are not included in this figure.

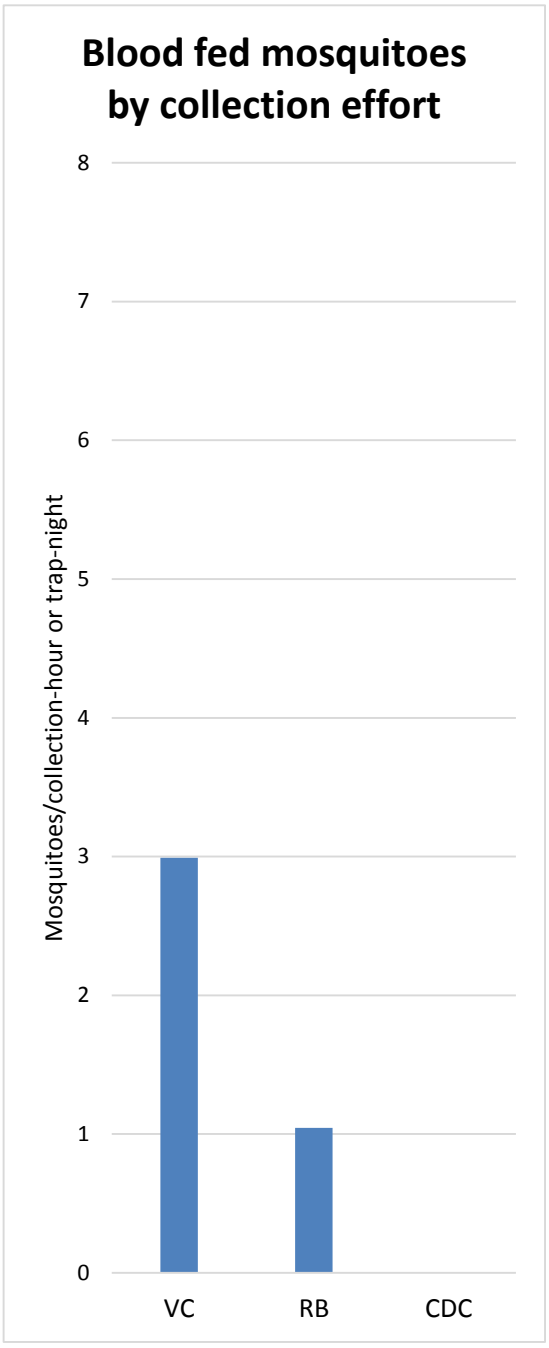

Figure 2.7: Blood fed mosquitoes collected per method, standardized by trap-nights (RB, CDC) or collection-hours (VC).

Table 2.5: Mammal blood meal hosts identified by DNA barcoding of wild-caught, blood-fed mosquitoes. *Red deer and moose blood meal hosts were likely misidentified white-tailed deer, and are included in this column.

	Eastern chipmunk	Eastern cottontail	Human	Raccoon	Virginia opossum	White-tailed deer*	*TOTAL*
Aedes canadensis	1	1	3			6	**11**
Ae. cinereus	3		1			6	**10**
Ae. fitchii/stimulans			1			5	**6**
Ae. stimulans						4	**4**
***Ae.* spp.**						4	**4**
Ae. triseriatus				2		2	**4**
Anopheles punctipennis			1			40	**41**
An. quadrimaculatus		1	3		3	28	**35**
Culex r/p/s			1			1	**2**
Cx. territans			1				**1**
Culiseta melanura			1			1	**2**
TOTAL	**4**	**2**	**12**	**2**	**3**	**97**	**120**

Table 2.6: Avian blood meal hosts identified by DNA barcoding of wild-caught, blood-fed mosquitoes.

	American robin	American woodcock	Black-capped chickadee	Blue jay	Eastern phoebe	Fish crow	Gray catbird	House sparrow	Northern cardinal	Red-eyed vireo	Red-winged blackbird	Song sparrow	Tufted titmouse	Wood thrush	TOTAL
Aedes canadensis									2					1	**3**
Ae. cinereus		1							2						**3**
Culex r/p/s				1	1	1		1					1	1	**6**
Culiseta melanura	2		1		2		1		3	2	2	4	1	1	**19**
Cs. morsitans											1		1	4	**6**
TOTAL	**2**	**1**	**1**	**1**	**3**	**1**	**1**	**1**	**7**	**2**	**3**	**4**	**3**	**7**	**37**

Table 2.7: Amphibian/reptile blood meal hosts identified by DNA barcoding of wild-caught, blood-fed mosquitoes.

	Gray tree frog	Spring peeper	Common snapping turtle	TOTAL
Aedes canadensis			1	**1**
Aedes cinereus	1			**1**
Ae. fitchii/stimulans	1			**1**
Cs. melanura	1			**1**
Cx. territans	4	1		**5**
TOTAL	**7**	**1**	**1**	**9**

Table 2.8: DNA barcoding of 3 µl blood aliquots of single species origin. Species origin determined through primer assay is indicated as "Long" or "Short." Base pair length of BLAST and BOLD queries is reported with match statistics. Match score represents maximum match score in BLAST, which is a measure of the number of consensus nucleotides and length of the query. The probability of the match occurring by chance is reported by the E value. Match failures are denoted by "x."

Species	COI primer	Sequence length (bp)	BLAST			BOLD
			Max. score	E value	Max. identity (%)	Specimen similarity (%)
Canis lupus familiaris	**Long**	x	x	x	x	x
Domestic dog	**Short**	274	499	$5x10^{-138}$	99	100
Homo sapiens	**Long**	611	1122	0	99	100
Human	**Short**	278	497	$2x10^{-137}$	99	100
Odocoileus virginianus	**Long**	610	1094	0	99	99.34
White-tailed deer	**Short**	271	453	$4x10^{-124}$	97	98.1
Falco peregrinus	**Long**	598	708	0	88*	90.75†
Peregrine falcon	**Short**	273	276	$8x10^{-71}$	84†	89.66†
Branta canadensis	**Long**	615	1098	0	99	100
Canada goose	**Short**	357	x	x	x	x
Corvus brachyrhynchos	**Long**	614	1079	0	99	98.66*
American crow	**Short**	274	484	$1x10^{-133}$	99	98.9*
Larus delawarensis	**Long**	616	979	0	95	97.96*
Ring-billed Gull	**Short**	274	486	$3x10^{-134}$	98	99.62*
Meleagris gallopavo	**Long**	628	749	0	99	99.51‡
Wild turkey	**Short**	x	x	x	x	x
Crotalus horridus	**Long**	630	x	x	x	x
Timber rattlesnake	**Short**	275	490	$3x10^{-135}$	99	99.26

†Accurate species matches always produced poorer match scores than congenerics

*Inaccurate matches to congeneric species produced equal or better scores

‡A match to *Milvus migrans* (black kite) had equal scores to wild turkey entries. Perfect alignment of this entry to wild turkey and poor match to other black kite entries suggests that it is a misidentified or contaminated database entry.

Table 2.9: Threshold of detection and identification of DNA barcoding analysis using COI long primer set for amplification and sequencing. Dilution and match statistics are presented for each species template.

	Domestic dog				Human				White-tailed deer			
Dil.	Seq. length	E val.	BLAST max ID	BOLD sim.	Seq. length	E val.	BLAST max ID	BOLD sim.	Seq. length	E val.	BLAST max ID	BOLD sim.
10^{-1}	x	x	x	x	613	0.0	99	100	606	0.0	98	98.7
10^{-2}	x	x	x	x	617	0.0	99	100	x	x	x	x
10^{-3}	x	x	x	x	758	x	x	x	x	x	x	x
10^{-4}	x	x	x	x	x	x	x	x	x	x	x	x
	American crow*				Timber rattlesnake				Ring-billed gull*			
Dil.	Seq. length	E val.	BLAST max ID	BOLD sim.	Seq. length	E val.	BLAST max ID	BOLD sim.	Seq. length	E val.	BLAST max ID	BOLD sim.
10^{-1}	615	0.0	99	99.18	x	x	x	x	616	0.0	98	99.16
10^{-2}	609	0.0	99	99.33	x	x	x	x	623	0.0	94	98.44
10^{-3}	x	x	x	x	x	x	x	x	718	$3x10^{-34}$	68	x
10^{-4}	x	x	x	x	x	x	x	x	x	x	x	x

x: Noisy, unmatchable sequence

* Congeneric species entries often produced equal or better matches

Table 2.10: Threshold of detection and identification of DNA barcoding analysis using COI short primer set for amplification and sequencing. Dilution and match statistics are presented for each species.

	Domestic dog				Human				White-tailed deer			
Dil.	Seq. length	E val.	BLAST max ID	BOLD sim.	Seq. length	E val.	BLAST max ID	BOLD sim.	Seq. length	E val.	BLAST max ID	BOLD sim.
10^{-1}	274	$1x10^{-138}$	99	100	279	$7x10^{-136}$	99	100	278	$8x10^{-116}$	96	94.25
10^{-2}	x	x	x	x	280	$4x10^{-123}$	96	98.49	x	x	x	X
10^{-3}	x	x	x	x	306	$7x10^{-37}$	100	98.81	x	x	x	X
10^{-4}	x	x	x	x	273	$7x10^{-106}$	99	100	x	x	x	X
	American crow*				Timber rattlesnake				Ring-billed gull			
Dil.	Seq. length	E val.	BLAST max ID	BOLD sim.	Seq. length	E val.	BLAST max ID	BOLD sim.	Seq. length	E val.	BLAST max ID	BOLD sim.
10^{-1}	270	$2x10^{-121}$	96	96.9	273	$3x10^{-135}$	99	99.26	273	$2x10^{-122}$	96	97.72*
10^{-2}	271	$1x10^{-124}$	97	96.28	274	$3x10^{-135}$	99	99.26	275	$3x10^{-120}$	97	96.53†
10^{-3}	x	x	x	x	x	x	x	x	190	x	x	X
10^{-4}	x	x	x	x	x	x	x	x	x	x	x	x

x: Noisy, unmatchable sequence

* Congeneric species entries often produced equal or better matches

† Accurate species matches produced poorer match scores than congenerics

Table 2.11: DNA barcoding matches (BOLD similarity %, BLAST max. identity %) of COI long (white background) and COI short (blue background) amplification and sequencing of dual host ratio mixes and reciprocals (2:1, 0.5:1, 1:1) of 3µl volume whole blood. Match results that provided scores for both hosts are listed in **bold**. Only accurate species-specific matches are represented by match scores. Samples of same host ratios and amplified by the opposite primer set can be found in the chart by matching segments of the same border color pattern (red to red, blue to blue, and dotted line to dotted line.

x: Noisy, unmatchable sequence, or no matches provided by a specific database

		1					
		Human	Ring-billed gull	White-tailed deer	Domestic dog	American crow	Timber rattlesnake
2	Human		Human (96.68, 89)	Human (99.63, 98)	Dog (x, 79)	**Crow (95.78, 83) Human (90.6, 83)**	Snake (87.76, 90)
	Ring-billed gull	Human (98.14, 91)		Gull (99.25, 98)	Gull (91.45, 82)	Gull (x, 90)	Gull (88, 78)
	White-tailed deer	Human (98.2, 95)	Gull (98.65, 96)		Dog (99.64, 99)	x	Snake (99.63, 99)
	Domestic dog	Human (100, 99)	Gull (98.61, 95)	Deer (82.15, x)		Dog (97.94, 91)	Dog (93.06, 81)
	American crow	x	Crow (94.62, 95)	Crow (98.98, 95)	Crow (99.56, 98)		**Snake (82.59, 85) Crow (x, 74)**
	Timber rattlesnake	Human (100, 99)	Gull (100, 99)	x	x	Crow (96.22, 96)	
1	Human		Human (97.08, 90)	Human (100, 99)	Dog (98.82, 82)	**Crow (95.78, 83) Human (90.6, 83)**	Snake (96.21, 95)
	Ring-billed gull	Human (96.53, 85)		Gull (99.3, 98)	Dog (93.97, 86)	Crow (98.13, 95)	Snake (96.55, 92)
	White-tailed deer	Human (100, 99)	Gull (98.07, 94)		Dog (99.63, 99)	Crow (99.63, 99)	Snake (99.63, 99)
	Domestic dog	Human (100, 99)	Gull (97.78, 95)	Deer (95.7, 86)		Dog (97.4, 83)	**Snake (89.79, 80) Dog (87.39, 75)**
	American crow	Crow (95.66, 88)	**Gull (92.8, 82) Crow (92.23, 84)**	Crow (98.79, 94)	Crow (96.38, 98)		Snake (90.73, 89)
	Timber rattlesnake	Human (100, 99)	Gull (99.32, 98)	Deer (97.19, 90)	Snake (x, 67)	Crow (97.93, 95)	
0.5	Human		Human (97.95, 94)	Human (99.63, 98)	Dog (98.51, 95)	Crow (98.84, 97)	Snake (98.86, 97)
	Ring-billed gull	Human (98.14, 91)		Gull (99.31, 99)	Dog (97.95, 92)	Crow (98.13, 97)	Snake (97.59, 91)
	White-tailed deer	Human (100, 99)	Gull (97.35, 93)		Dog (100, 99)	Crow (99.64, 99)	Snake (98.91, 99)
	Domestic dog	Human (100, 99)	Gull (97.21, 93)	Deer (99.13, 94)		**Crow (x, 80) Dog (x, 80)**	Snake (96.55, 94)
	American crow	**Human (94.54, 87) Crow (x, 83)**	Gull (95.04, 92)	Crow (98.97, 93)	Crow (99.57, 99)		Snake (88.72, 90)
	Timber rattlesnake	Human (100, 99)	Gull (99.32, 98)	Deer (98.75, 93)	x	Crow (98.14, 96)	

DNA barcoding of single species 3 µl aliquots with the COI long primer set most often matched accurate database entries (Table 2.8). *Falco peregrinus* (peregrine falcon) and *Larus delawarensis* (ring-billed gull) samples often matched equally or better to inaccurate but congeneric species. Barcoding with COI short primers matched human (*Homo sapiens*) and ring-billed gull accurately and with high match statistics, but peregrine falcon identification produced low match statistics, and Canada goose (*Branta canadensis*) and wild turkey (*Meleagris gallopavo*) amplification and sequencing produced data of too poor quality to be identified.

Thresholds of detection determined by DNA barcoding of ten-fold serial dilutions amplified with the COI long primer set are shown in Table 2.9, and COI short threshold assays in Table 2.10. COI short primer assays produced poor quality sequence and match failures for all dilutions of *B*. *canadensis*, *L*. *delawarensis*, and *M. gallopavo*, and so are not included. *F*. *peregrinus* serial dilutions amplified by COI long or short primers were mostly unmatchable by BLAST, except for 10^{-3} dilution template amplified with COI long primers, for which *F*. *peregrinus* was included in results but was not the top match. A general trend was for greater or equal amplification success by COI short primers than COI long, and often at more dilute amounts of template.

Amplification and identification of systematically mixed 3 µl volume blood samples by COI long and COI short primers exhibited identification bias of each set for or against certain hosts. For example, mixes containing timber rattlesnake blood were more likely to be identified as timber rattlesnake using COI short primers (13/15 times) than with COI long (1/15 times)

(Table 2.11). Human DNA in mixes was more frequently identified with COI long primers (13/15) than COI short (8/15). There were seven mixed blood samples from which both hosts could be identified by a single sequence query of BOLD and/or BLAST, and 29/45 samples which were identified differently by one primer set than the other.

Discussion

Blood meal analyses of mosquitoes collected in Ithaca, NY over the summer of 2012 indicate potential avenues of disease transmission as feeding patterns connecting avian, mammal, and reptile/amphibian wildlife populations and humans. *Culex pipiens* are competent vectors of WNV (Kilpatrick et al. 2005, Hamer et al. 2008), and *Cx*. restuans/pipiens/salinarius were shown by my methods to feed on a variety of avian species as well as humans and white-tailed deer, demonstrating their importance for local transmission if WNV were introduced from nearby counties (http://www.health.ny.gov/diseases/west_nile_virus/). Other arboviral diseases such as St. Louis encephalitis could also be maintained in avian-mosquito-mammal networks.

Aside from white-tailed deer, house sparrows, blue jays, and fish crows, most hosts I identified play unknown roles in mosquito-borne disease ecology, and matching hosts and mosquito species provides a basis for future studies in this field. Due to their abundance, peridomesticity, and seroconversion in response to WNV exposure, Virginia opossum (*Didelphis virginianus*) and raccoons (*Procyon lotor*) are candidate WNV sentinel animals, but their status as reservoirs of infectious virus is unresolved (Root 2012). *Ae. canadensis* and *An. quadrimaculatus* hosts included both humans and Eastern cottontail rabbits (*Sylvilagus*

floridanus), which have developed viremia adequate for WNV transmission to mosquitoes under experimental conditions (Tiawsirisup and Platt 2005), but exhibit very low seroprevalence in the wild (Root 2012). Consequently, host feeding patterns and infection rates in these species should be investigated further.

My traps and aspirators performed well as surveillance tools assessing vector diversity and targeting blood fed mosquitoes. In pilot studies without the use of resting boxes, blood fed *Anopheles* were not captured. In this study, they represent the majority of my blood fed collections. I recommend the use of multiple collection methods in combination to provide broader coverage of mosquito species diversity and avoid a strong bais, as was evident from species distribution and blood fed mosquito biases in my collections standardized by effort (Figs. 2.2-2.5, 2.7). Vegetation aspirators and resting boxes were the most effective modes to target the resting, blood fed population. Vegetation aspirators collect greater numbers of blood fed mosquitoes per collection-hour than resting boxes catch per trap-night; however, labor and personnel restrictions may lead to deployment of RB traps in some studies since aspirating mosquitoes from resting boxes once a day is less labor intensive than operating a large vegetation aspirator an hour per day. Species bias must also be taken into account since resting boxes and vegetation aspirators caught different species in areas where they were used simultaneously (Figs. 2.2-2.4).

Blood meal analysis of wild-caught mosquitoes revealed candidate vector species that could pose an epidemiologic threat due to their frequency of blood feeding on both humans and other animals, and/or their propensity to be generalistic feeders across avian, mammal, and amphibian/reptile hosts. Evidence-based vector control could selectively target vector species

according to the transmission ecology of mosquito-borne diseases at risk of being introduced to the area. *Culiseta melanura* could be a target for vector control, since its hosts spanned 15 species across mammal, avian, and reptile/amphibian taxa, and it has been implicated in maintaining EEE infections in the wild bird population, although other mosquito species, such as *Cx. restuans, Cx. pipiens,* and *Cx. salinarius*, are likely bridge vectors to humans and other mammals (Armstrong and Andreadis 2010). I cannot conclude whether low collection rates of significant vector species are simply due to an inability to target them efficiently. The relative abundance of these species may be higher in wild populations than is suggested by the species distribution of my collections.

Although no identified blood meals were from heartworm hosts, continued sampling in areas of high dog activity could be one component of heartworm transmission surveillance in conjunction with screening vector mosquitoes for infective stage larvae. Tompkins County prevalence during the collection year was 0.23% (http://www.capcvet.org/parasite-prevalence-maps/); however, neighboring counties with higher prevalence and imported dogs from the Southeastern US pose a constant disease risk to non-endemic areas.

Blood meal analysis can disentangle transmission patterns, incriminate key vector species, and identify reservoirs of infection. Care must be taken when interpreting the biological significance of blood meal analysis sequence data, even when processing data and matching sequences to hosts. My conclusions from quality control analyses are that match thresholds, success rate, and bias should be defined per primer set, database, host, and laboratory technique.

Some primer host bias was evident in my analysis, and most mixed blood samples identified as one host with COI long primers and another by COI short (Table 2.11). In rare cases, both blood meal hosts could be identified by a single query of BOLD or BLAST, but this was only possible for hosts from divergent taxa such that results presented two clear groups of high probability matches. These conditions are unlikely to occur in digested or degraded blood in wild-caught mosquitoes, but detection of primate sequence in addition to other animals could be considered evidence of human blood in areas where no other primates are present. To my knowledge this is the first description of multiple blood meal hosts being identified by a single query of DNA barcode databases. In my collection, two mosquito blood meals were identified as different hosts with subsequent sequencing, one bird:bird and one deer:frog. Wild-caught blood meals such as these could be analyzed with different primer sets or cloned if higher confidence identification of multiple species is required.

Detailed record keeping of database match statistics could pinpoint species identifications that consistently present problematic database matches. An example from my analysis is white-tailed deer identifications. DNA barcoding of wild-caught deer host blood meals sometimes matched to cervids not found in my study sites or the US. Quality control analysis with serial dilutions of local deer blood allowed us to determine that matches to red deer, mule deer, or moose were highly likely to be partially digested white-tailed deer blood meals.

As discussed by Townzen et al. (2008), intelligent synthesis of sequence matches, database quality, and knowledge of local wildlife presence must guide blood meal analyses with molecular methods. Recommendations for future studies depend on the purpose and resources available for blood meal analysis by DNA barcoding. If laboratory resources must be conserved,

COI short primers may be effective without COI long primers since a higher match success rate was achieved overall, and blood samples were identifiable at higher dilutions than with COI long primers. If laboratory resources are not a limiting factor, blood meals could first be analyzed with COI long primers, and samples unsuccessful during matching, sequencing, or amplification can then be analyzed with COI short primers (Townzen et al. 2008). If unusual or uncommon hosts are identified, investigators could benefit from compiling a local database from DNA template obtained from local hosts suspected to have problematic sequences for DNA barcoding. Using blood for compiling a local database would be most accurate in recreating reaction conditions of DNA barcoding wild samples, but tissue samples may be easier to obtain and would provide greater quantity and quality of template DNA. Serial dilutions and other quality control analyses could indicate errors in sequence matching and public database entries.

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CHAPTER THREE

Fine-scale temperature fluctuation modulation of *Dirofilaria immitis* development in *Aedes aegypti*

ABSTRACT

This study evaluated the validity of standard heartworm development unit (HDU) degree-day predictions of *Dirofilaria immitis* extrinsic incubation period under constant and fluctuating temperature treatments of equal average daily temperature. Liverpool strain *Aedes aegypti* mosquitoes were infected with *D. immitis* microfilariae and dissected to determine development rate into L3 stage larvae. Time until L3 development in Malpighian tubules and detection in mosquito heads was shorter for larvae experiencing a daily regime of 19±9°C than larvae at constant 19°C; however larvae in fluctuating regimes that remained above the 14°C threshold exhibited longer time until L3 development in Malpighian tubules than larvae developing under constant temperatures, and there was no significant difference in detection in mosquito heads. My results demonstrate that hourly accumulation of HDUs more accurately predicts first detection of L3 stage *D. immitis* in mosquito heads, but development time is significantly different between fluctuating and constant temperature treatments spanning the 14°C development threshold, implicating a physiological basis for these development differences. I suggest that average daily temperature models underestimate L3 development in colder temperatures, and spatio-temporal models of *D. immitis* development and transmission risk should use hourly temperature data when investigating high daily temperature ranges or fluctuations across 14°C.

INTRODUCTION

Temperature and extrinsic incubation period (EIP) are basic parameters in predictive models of vector-borne disease transmission risk and seasonality. *Dirofilaria immitis* EIP is defined by heartworm development units (HDUs) that factor average daily temperature above *D. immitis* minimum temperature for development, 14°C, and are calculated as follows:

$$\sum Average\ daily\ temp - 14℃ = Accumulated\ HDUs$$

Previous studies have shown that ingested microfilariae mature to infective stage L3 larvae and migrate to the mosquito head and labium once 130 HDUs have been accumulated (Fortin and Slocombe 1981). This formula provides the basis for defining risk across geographic regions and peak periods of dog heartworm transmission in the United States and other countries (Knight and Lok 1998, Vezzani and Carbajo 2006, Medlock et al. 2007, Genchi et al. 2009, Cuervo and Fantozzi 2013).

Although HDU predictions have proven useful on a broad scale, studies with other vector-borne pathogens suggest that the relationship between temperature fluctuation and pathogen or parasite incubation is not linear (Cornel et al. 1993, Reisen et al. 1993, Arthurs et al. 2003, Paaijmans et al. 2009, 2010). The majority of supporting literature for this phenomenon focuses on extrinsic incubation dynamics of arboviruses and malaria, but *Thripinema nicklewoodi*, a parasitic nematode of thrips, reared at a temperature regime of 35:20°C for 14:10 h was shown to develop similarly to *T. nicklewoodi* reared at a constant 20°C (Arthurs et al. 2003) despite the 28.75°C daily average of the fluctuating regime. Temperature

fluctuation has been shown to modulate extrinsic incubation period and R_0 of malaria as discussed in depth by Paaijmans et al. (2010). The authors implicate non-linear effects of temperature fluctuation for discrepancies between malaria transmission predictions and actual caseload in areas that do not meet degree-day developmental thresholds. Interactions between fluctuation amplitude and baseline average temperature produced non-linear changes in EIP and mosquito survival and, therefore, mosquito vectorial capacity for malaria (Paaijmans et al. 2009).

Daily average approximation of temperature experienced by vectors and disease agents ignores biological effects of fluctuation and mathematical discrepancies of prediction models at finer resolutions, and is unrealistic given that mosquitoes occupy spatiotemporal habitats with distinct environmental conditions (Meyer et al. 1990). Microhabitats vary with ecological niches of mosquito species in question, and studies monitoring microhabitat conditions compared to average ambient conditions conclude that a mosquito could experience a "composite thermal environment" resulting in a different EIP than that calculated by average ambient daily temperature (Meyer et al. 1990).

This is the first study to examine the effects of daily temperature fluctuation on development and transmission of *D. immitis*, and there has been little investigation of the accepted HDU model assumptions, or the likelihood of geospatial shifts in transmission risk due to climate change. To address these issues, I exposed *D. immitis*-infected *Ae. aegypti* to temperature regimes representing fluctuations recorded by weather monitoring stations from July-August, 2012 in US counties with high dog heartworm prevalence in the same year (

http://www.capcvet.org/parasite-prevalence-maps/). An additional 10:28°C temperature regime tested effects of fluctuation above and below 14°C, the accepted minimum temperature for development of *D. immitis* in the mosquito (Fortin and Slocombe 1981). By my hypothesis, HDU hourly accumulation could be expressed:

$$\sum Hourly\ temp\ observation - 14℃ = Accumulated\ HDUhrs$$

130 HDUs are equivalent to 3120 HDUhrs, and mosquitoes in the 10:28°C regime will accumulate 168 HDUhrs/day in contrast to 120HDUhrs/day under constant 19°C. This should result in infective stage *D. immitis* detection in mosquito heads/proboscises as much as 7.43 days earlier in fluctuating treatment compared to constant 19°C. Infected mosquitoes were dissected to compare *D. immitis* development timepoints and differences in degree-day model predictions and transmission potential between treatments were analyzed.

Materials and Methods

Mosquito rearing Liverpool strain *Aedes aegypti* were acquired as eggs from Cheri-Hill Kennel & Supply Inc. Stanwood, MI. Mosquitoes were maintained in an environmental chamber set at a temperature of 29˚C, 90% RH and 10 h L: 10 h D photo-regime with 2 h periods of dusk and dawn. Adults were held in a 30-cm^3 screened cage and provided with 10% sucrose ad lib. Two- to five-week-old chicks were offered twice to three times a week for 20 mins at each blood feeding.

Eggs were collected on paper towels lining oviposition vessels placed inside the cage. Papers with deposited eggs were air dried for 24 h and stored at 100% RH for at least seven

days and up to a month before hatching. Egg papers were soaked in a flask filled with 750 ml of water for 30 min before applying vacuum pressure for 45 min for hatching. Vacuum pressure was released and 30 mg of ground larval diet added. Larvae were held overnight in the flask and transferred into trays the next day.

Larvae were transferred to 3.9-liter plastic trays (21 by 21 by 7 cm) containing 1 L of purified water and secured with mesh tops. Larvae were added 200 per tray. Larval diet (Hikari® Cichlid Gold Large Pellet; Kyorin Co. Ltd. Himeji, Japan) was added to each tray according to the following regime modified from Mclean-Cooper et al. (2008): day 1, 30 mg (ground fish pellet); day 2, 30 mg (ground fish pellet); day 3, 582 mg (3 whole pellets and ground pellets). Suitability of this diet was analyzed in pilot studies.

Handling of microfilaremic blood Microfilaremic and non-infected blood was supplied by Dr. Byron Blagburn (Auburn University, AL) and Cheri-Hill Kennel & Supply Inc. (Stanwood, MI) in overnight shipments of heparinized collection tubes. Microfilaremic blood was pooled together and used no later than 7 hrs after receiving shipments.

Determining mf density Blood microfilarial density was determined immediately prior to each infection. Microfilariae in 3 x 20 µl aliquots of blood were counted from the pooled blood sample. Counting was performed using a modified method based on Theis et al. (2000). Briefly, each 20 µl aliquot of infected blood was diluted in 40 µl of water and held at room temperature for 20 mins. Diluted aliquots of blood were each spread evenly over three microscope slides for scanning under a phase contrast microscope at 100X. Only moving microfilariae were counted. After determining the density of live microfilariae, heartworm-

negative dog blood was added to attain the desired microfilarial density of 3500 microfilariae/ml.

Infecting mosquitoes Cups holding an estimated 200 pupae were placed inside 7L plastic containers secured with mesh in which they were allowed to eclose. Moistened sugar cubes were provided on tissue over the mesh lid. Sugar and water were removed 15-20 h before offering microfilaremic blood. On the day of infection, 7L plastic containers of 3- to 5-day-old mosquitoes were chilled at 4˚C for 12 min for immobilization. Females were placed 100-200 mosquitoes per 7L plastic container; males were discarded. Microfilaremic blood was offered according to the methods of Lai et al. (2000) with the following modifications. Known densities of microfilariae in heparinized dog blood were added to glass feeders secured at the base with washed hog intestine (Syracuse Casing Company, Syracuse, NY). Blood was warmed in the feeder apparatus using a circulating water bath set at 37˚C (Harrington et al. 2001). Feeders were placed on mesh carton lids, and mosquitoes were allowed to feed on infected blood for 30-60 min. A plastic disposable 3 ml pipette was used to mix blood within the feeders every 4-7 min to prevent microfilariae from settling unevenly on the feeding membrane (Kartman 1953). A subset of mosquitoes from the same cohort were kept separate and offered human blood. These mosquitoes were kept in the same growth chamber treatments for mortality comparison as controls. After blood feeding, chilled, immobilized mosquitoes were sorted to keep only fully engorged females.

Blood fed females were maintained in plastic containers and held at 90% RH and at constant or fluctuating temperature regimes (described above).

Monitoring *D. immitis* development Three to five mosquitoes were frozen immediately after feeding and dissected to verify mean microfilariae ingested per mosquito. Mosquitoes were dissected at timepoints beginning before 130 accumulated HDUs and continued until the end of the experiment. At each timepoint, heads and abdomens of cold-immobilized females were dissected in separate drops of saline on glass microscope slides to be inspected visually for presence and staging of *D. immitis* larvae (Taylor 1960, Lai et al. 2000, Tiawsirisup 2007). Data recorded per mosquito included number, developmental stage, and mosquito body region/organ in which larvae were found.

Treatment conditions All mosquitoes experienced a 10 h L: 10 h D photo-regime with 2 h periods of dusk and dawn, temperature regimes were as follows:: 19°C, 19±9°C, 22° C, 22±4°C, 26°, and 26±4°C.

Data Analysis

Number of *D. immitis* at each developmental stage was recorded as well as the timepoint of first detection of infective stage L3 larvae in mosquito heads. Kaplan-Meier log-rank statistic was used to compare the distribution of L3 detection in mosquito Malpighian tubules and heads as plotted against timepoints measured in HDU and HDUhrdays. HDUhrdays were calculated by dividing HDUhrs by 24 to convert to units more directly comparable to HDUs. Development times were compared between treatments of equal baseline average temperature.

Results

First detection of L3 *D. immitis* is presented for each treatment in HDUhrs/24, HDU, and days post-infection (Table 3.1). HDUhrs/24 and HDU measurements are equal for treatments maintained above 14°C, but they diverge when calculated for 19±9°C.

L3 detection in Malpighian tubules and L3 detection in heads were analyzed separately as outcome events of Kaplan-Meier survival analyses comparing fluctuating treatments with constant temperature treatments of the same baseline average temperature using a log-rank test of significance; Figures 3.1-3.8 display these results as rates of detection over dissection timepoints. Analyses comparing 19±9°C and 19°C obtained development estimates using HDU and HDUhrsdays, which are calculated as HDUhrs/24; HDUhrsdays were equal to HDU measurements for all other analyses, therefore, only HDUhrsdays are shown. Descriptive statistics of these analyses can be found in Appendix C.

Detection rate curves of larvae in Malpighian tubules were all significantly different between fluctuating and constant treatments of the same average daily temperature (df=1, $p<0.001$) (Figures 3.1-3.4). L3 *D. immitis* in Malpighian tubules were detected later in fluctuating treatments than in constant treatments (Figures 3.3 and 3.4), except in 19±9°C and 19°C where L3 were instead detected earlier under fluctuating temperature than under constant 19°C (Figures 3.1 and 3.2).

Table 3.1: Infection date, temperature recordings, *D. immitis* sample size, and timepoints of first detection of L3 *D. immitis* in Malpighian tubules and heads are presented for each treatment replicate. Timepoints for which HDUhrs/24=HDU are respresented by "-" in the HDU column. "x" marks cells with no observations.

Treatment	Inf. date	Avg. temp (St. dev)	Worm count	First L3 in Malpighian tubules		Days post infection	First L3 in heads		Days post infection
				HDUhrs/24	HDU		HDUhrs/24	HDU	
19°C	10/2/13	19.22 (0.27)	251	125.25	-	24	x	x	x
	8/6/13	19.09 (0.37)	212	131.36	-	26	131.36	-	26
19±9°C	10/2/13	19.34 (8.58)	104	127.60	101.81	19	127.60	101.81	19
	8/6/13	19.04 (8.68)	172	116.00	86.96	17	122.76	91.96	18
22°C	8/21/13	21.7 (0.38)	180	122.23	-	16	138.33	-	18
22±4°C	8/21/13	20.79 (4.47)	170	122.29	-	18	146.38	-	19
	5/29/13	21.91 (3.89)	422	135.45	-	17	134.04	-	17
26°C	10/9/13	25.40 (0.36)	153	130.60	-	12	137.68	-	12
	8/21/13	25.37 (0.57)	38	100.61	-	9	x	x	x
26±4°C	10/9/13	26.03 (3.87)	183	130.67	-	11	130.67	-	11
	5/29/13	26.11 (3.77)	363	118.11	-	10	132.19	-	11
	8/21/13	25.42 (4.37)	195	126.38	-	11	126.38	-	11

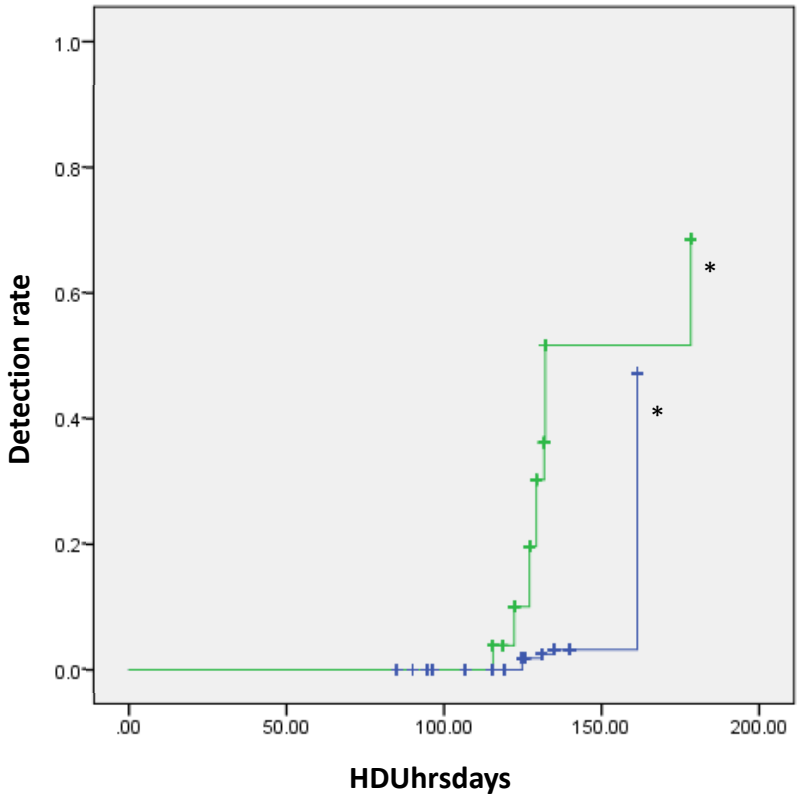

Figure 3.1: L3 detection rate in Malpighian tubules is plotted against HDUhrsdays timepoints for both 19±9°C (green) and 19°C (blue) treatments.
*Curves were significantly different by the logrank test (df=1, p<0.001).

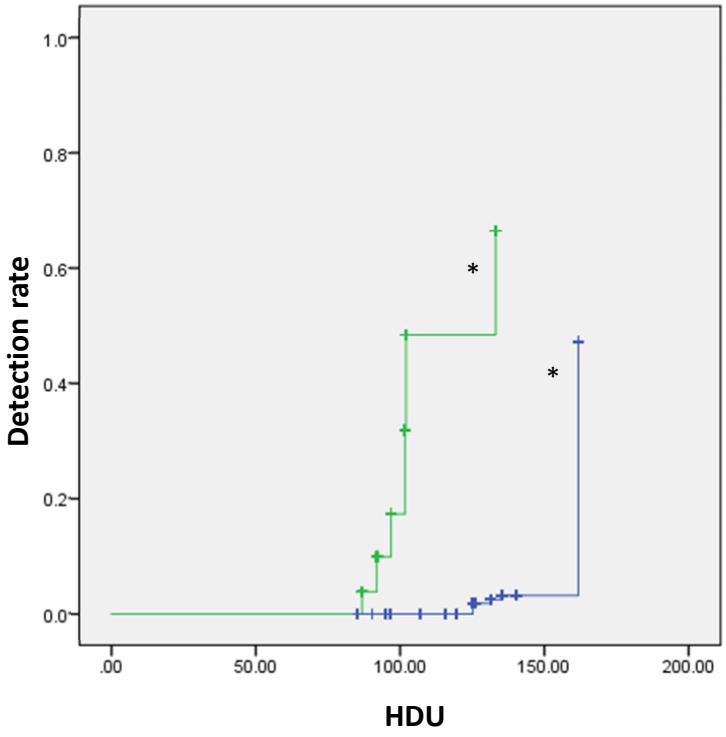

Figure 3.2: L3 detection rate in Malpighian tubules is plotted against HDU timepoints for both 19±9°C (green) and 19°C (blue) treatments.
*Curves were significantly different by the logrank test (df=1, p<0.001).

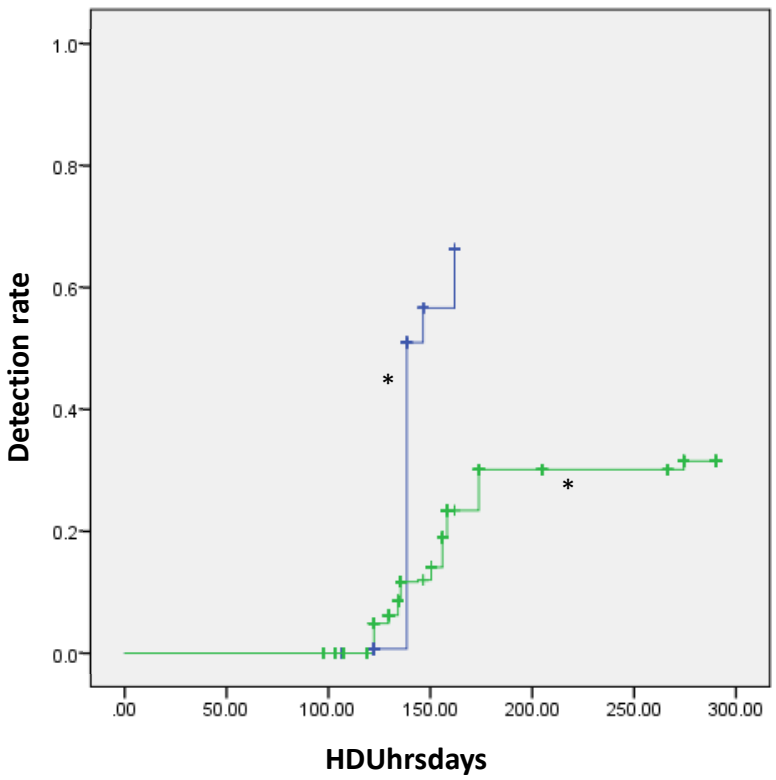

Figure 3.3: L3 detection rate in Malpighian tubules is plotted against HDUhrsdays timepoints for both 22±4°C (green) and 22°C (blue) treatments.
*Curves were significantly different by the logrank test (df=1, p<0.001).

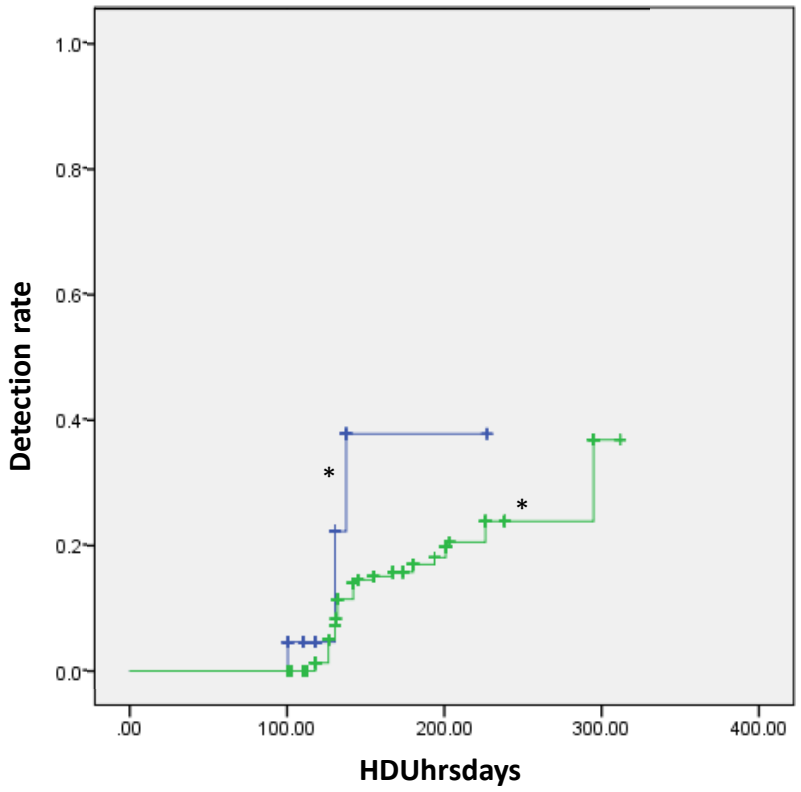

Figure 3.4: L3 detection rate in Malpighian tubules is plotted against HDUhrsdays timepoints for both 26±4°C (green) and 26°C (blue) treatments.
*Curves were significantly different by the logrank test (df=1, p<0.001).

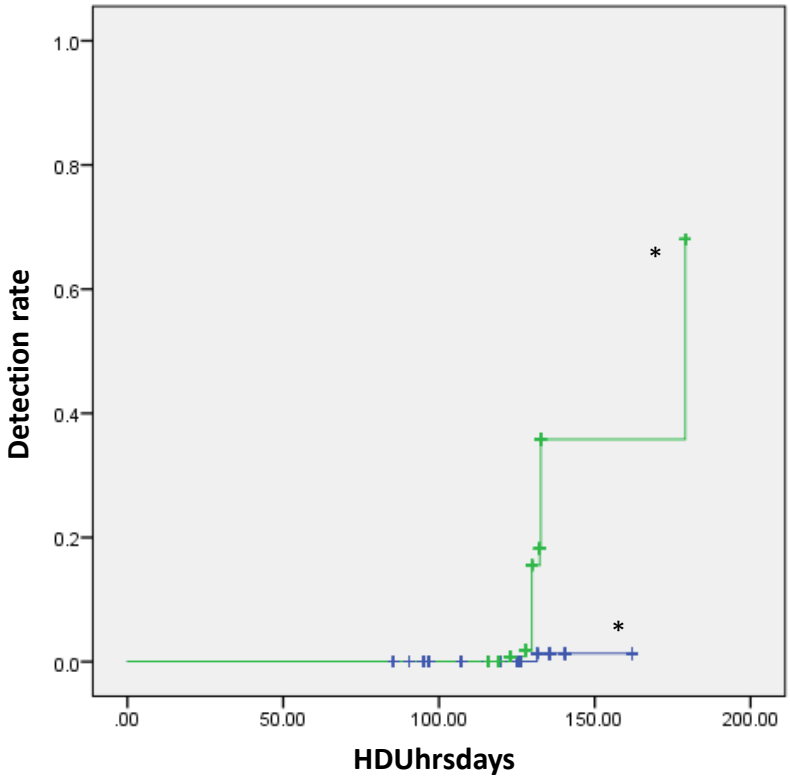

Figure 3.5: L3 detection rate in mosquito heads is plotted against HDUhrsdays timepoints for both 19±9°C (green) and 19°C (blue) treatments.
*Curves were significantly different by the logrank test (df=1, p<0.001).

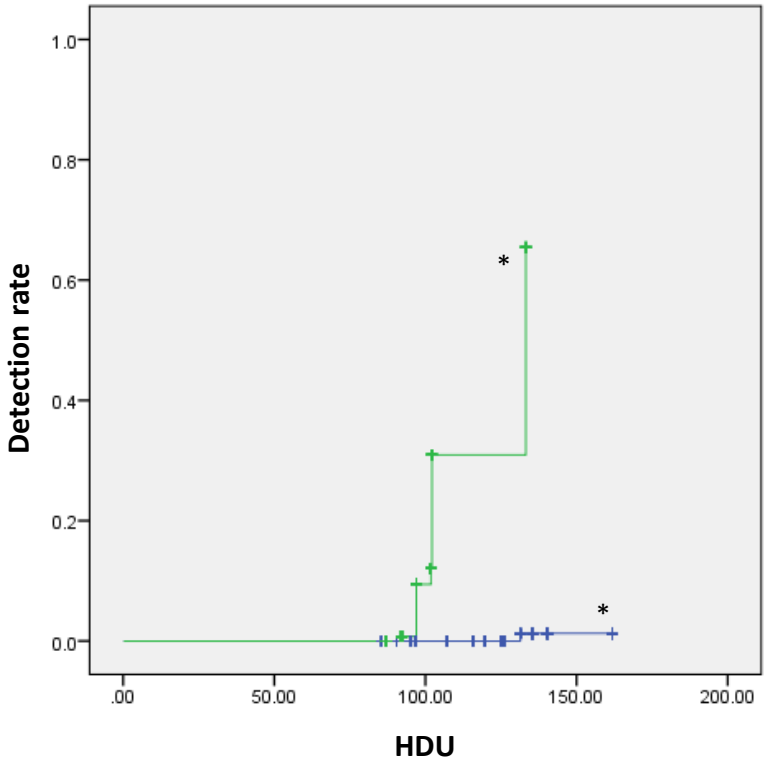

Figure 3.6: L3 detection rate in mosquito heads is plotted against HDU timepoints for both 19±9°C (green) and 19°C (blue) treatments.
*Curves were significantly different by the logrank test (df=1, p<0.001).

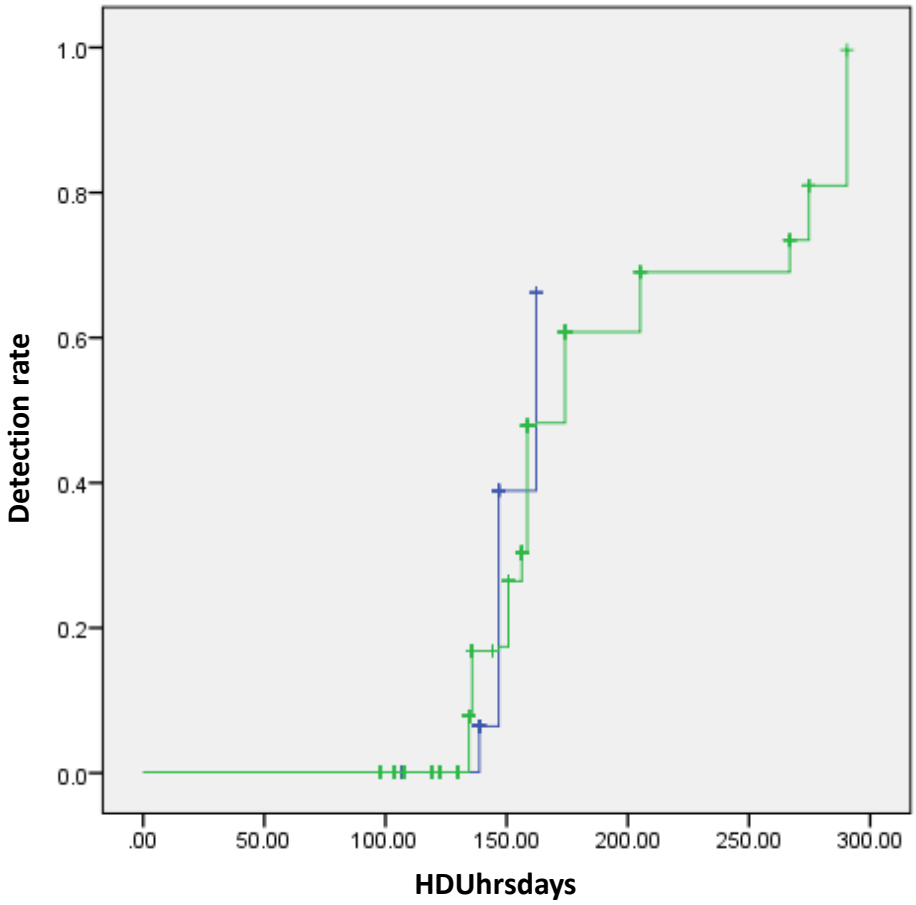

Figure 3.7: L3 detection rate in mosquito heads is plotted against HDUhrsdays timepoints for both 22±4°C (green) and 22°C (blue) treatments.
Curves were not significantly different (p=0.825).

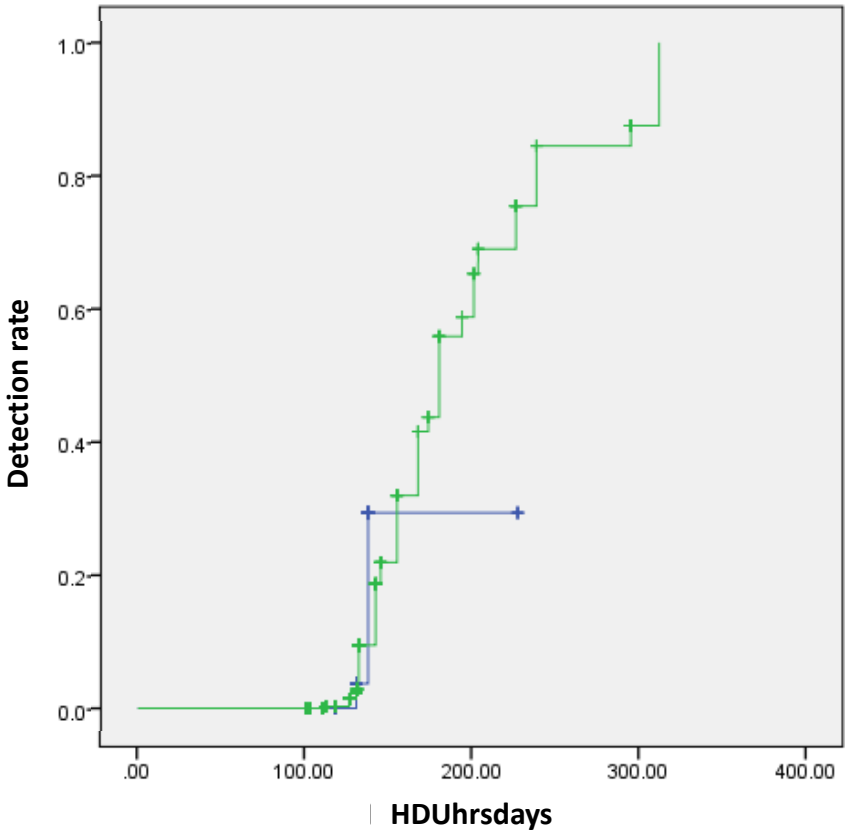

Figure 3.8: L3 detection rate in mosquito heads is plotted against HDU timepoints for both 26±4°C (green) and 26°C (blue) treatments.
Curves were not significantly different (*p*=0.506).

L3 *D. immitis* were detected in mosquito heads in 19±9°C treatments at earlier timepoints than 19°C when compared across HDU (df=1, $p<0.001$) or HDUhrsdays (df=1, $p<0.001$) (Figures 3.5 and 3.6). L3 detection did not differ significantly in comparisons of other fluctuating and constant treatments (Figures 3.7 and 3.8).

Discussion

Spatiotemporal models of transmission risk define *D. immitis* development periods as time windows no longer than 30 days during which 130 HDUs can be accumulated (Knight and Lok 1998, Sacks et al. 2004). HDUs are calculated using either average daily temperature or minimum daily temperature to determine earliest and latest transmission potential each year (Vezzani and Carbajo 2006, Genchi et al. 2011). Assumptions underlying these approaches are: 1) infected mosquito longevity in the field is approximately 30 days no matter what season of the year; 2) infective stage *D. immitis* at the beginning of each season develop from microfilariae ingested by the vector during the same uninterrupted development period (i.e., not carried over from before winter or a long interruption of transmission season); and 3) development thresholds can be represented by average daily temperature (often calculated as *maximum temperature + minimum temperature/2*) or minimum daily temperature.

My results suggest that modeling low temporal resolution temperature data such as daily average or daily minimum temperature can underestimate *D. immitis* development rate and range of transmission. *Dirofilaria immitis* development predictions were more accurate when calculated with hourly temperature observations rather than average daily temperature. Seemingly small discrepancies between average daily and hourly temperature accumulations

compounded to the extent that infective stage larvae experiencing 19±9°C were first detected in mosquito heads eight days earlier than *D. immitis* under constant 19°C (Table 3.1). Furthermore, first L3 larvae detected in mosquito heads at 19±9°C had only accumulated 101.81 and 91.96 HDUs calculated by average daily temperature—much earlier than the standard 130 HDU model predictions; however, if accumulated degree-days are instead calculated by hourly temperature observations above threshold, these larvae actually accumulated 127.60 and 122.76 HDUhrs/24. Average daily temperature HDU calculations were demonstrated to underestimate *D. immitis* development under my fluctuating condition spanning the 14°C development threshold, and a minimum daily temperature estimation of HDUs would consider *D. immitis* development impossible under this regime's 10°C minimum daily temperature.

Survival analysis of *D. immitis* development in mosquito Malpighian tubules showed that 19±9°C larvae developed to L3 stage faster than larvae at 19°C, even when using an hourly calculated HDU scale (Figures 3.1 and 3.2). This suggests that there is a biological difference in development rate between these two treatments that is not accounted for solely by degree-day differences. Treatments that fluctuated above threshold showed the reverse pattern: time to L3 development was longer for larvae experiencing temperature fluctuation than larvae under constant temperature (Figures 3.3 and 3.4). One hypothesis is that, for natural populations of *D. immitis*, experiencing temperature fluctuation under developmental threshold may be a trigger for late-season developing larvae to curtail their normal L2-L3 stadium in favor of increasing the likelihood of transmission into a primary vertebrate host where environmental conditions will have a much smaller impact on survival. In contrast,

temperature fluctuations that remain above threshold may not be enough of a signal to curtail development, and may instead delay development to L3 stage by increasing physiological resources allocated to adjusting growth to unstable environmental conditions. That the fluctuating temperature delay in Malpighian tubule development did not extend to significant differences in migration to mosquito heads may be due to low sample size within the time period of developmental difference, or it is possible that other amplitudes of temperature fluctuation could have a greater effect, as has been shown for similar studies of malaria extrinsic incubation period and climate (Paaijmans et al. 2009, 2010).

The extent to which the effects observed in my study are generalizable to wild populations of *D. immitis* and mosquito vectors requires further study. *Aedes aegypti* is not a common vector of *D. immitis* across most of the US, and susceptibility and competence of mosquito vectors for *D. immitis* will vary within and between vector species and, possibly, between populations of *D. immitis* (Bradley et al. 1990, Tiawsirisup 2007, Ledesma and Harrington 2011). My infections only tested doses of 3,500 microfilaria/ml, and the dynamics of typical microfilarial densities of infectious blood meals taken by wild mosquito vectors are undescribed and would likely vary depending on whether the reservoir population is stray dogs, owned dogs, or wild canids. Actual temperature fluctuations experienced by vectors and their parasites would also depend not only on ambient air temperature, but on microhabitats sought out during periods of activity and rest (Meyer et al. 1990). Physiological differences between geographical isolates of *D. immitis* may contribute to developmental responses to temperature fluctuations of different amplitude or baseline average temperature than is typical of their location. Extrinsic incubation period developmental differences were observed by Ernst and

Slocombe (1983) when rearing Georgia and Ontario *D. immitis* strains under low temperatures, and, in their preliminary trials, Georgia strain larvae exhibited higher mortality than Ontario strain larvae when exposed to 14°C, and Ontario larvae remained at L2 stage for a longer duration than Georgia strain when reared at a constant temperature of 26°C. Although their trials were focused on the effect of four to eight days of exposure to low temperatures during extrinsic incubation period, their conclusions support the hypothesis that temperature changes during extrinsic incubation period alter *D. immitis* development in ways which are ignored by the standard HDU model, and that model characteristics may have to account for developmental adaptations of regional *D. immitis* populations.

Surveillance and prevention measures informed by underestimates of dog heartworm range and transmission period could hinder efforts to limit emergence of the disease to naïve domestic dog and wildlife populations in areas of colder climate. Sacks and others (2004) constructed a model of dog heartworm prevalence that accurately identified areas of high and medium prevalence by incorporating climate data, coyote serology and carcass inspection, vegetation cover, and precipitation; however, actual prevalence in predicted low-prevalence areas was underestimated. Slocombe and others (1989) developed isolines circumscribing regions sharing the same start or end dates of heartworm transmission season as determined by HDU accumulation. By their methods and comparisons with laboratory trials of *D. immitis* extrinsic incubation under constant low temperatures, it was determined that 130 HDUs was a conservative estimate for L3 development, and that average temperatures below 14°C would be unsuitable for L3 development since no HDUs would be accumulated. Based on the model assumption that mosquitoes live 30 days in the wild, they also propose that mosquitoes living

longer than 30 days—as assumed by the model—may explain early season transmission during average daily temperatures that are too low for L3 development within typical mosquito lifespans. My results provide an alternate explanation: *D. immitis* extrinsic incubation and, therefore, transmission is underestimated by HDUs calculated by average daily temperature. Consequently, isolines underestimate transmission season by not taking into account temperature fluctuation around average daily temperatures at or below 14°C. The current isolines would have to be redrawn to determine more accurate start and end dates during which hourly calculations of HDUs could allow for *D. immitis* L3 development.

My results suggest that there are mathematical and biological reasons that defining low-risk areas with average daily temperature models may be inappropriate. Furthermore, as climate change affects weather predictability and stability, the impact of fine resolution temperature fluctuation on vector-borne disease transmission becomes increasingly important. Weather station data usually include finer resolution temperature observations than daily averages, and new insights could be gained from incorporating these measures into degree-day algorithms predicting transmission risk, or developing separate evaluation methods for low-risk areas.

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CHAPTER FOUR

Entomological and socio-behavioral components of dog heartworm (*Dirofilaria immitis*) prevalence in two Florida communities

ABSTRACT

A knowledge, attitudes, and practices (KAP) questionnaire was administered alongside container surveys of potential mosquito breeding sites to residents of neighborhoods of two northern Florida counties of low and high historical dog heartworm (*Dirofilaria immitis)* prevalence. Adult mosquito collections were also conducted in each neighborhood for *D. immitis* screening and key vector incrimination. Mosquito and heartworm knowledge, disease risk concern, administration of heartworm prevention, and mosquito reduction practices were compared between neighborhoods, education levels, and pet ownership. Responses revealed that most residents were unaware that dog heartworm is transmitted by mosquitoes, and dog owners were more likely to be highly concerned about dog heartworm, know that mosquitoes transmit the disease and breed in standing water, and be willing to pay for and have accurate estimates of the cost of dog heartworm treatment. The majority of pet owners administered preventive drugs to their pets, and primary reasons for not administering prevention was risk unawareness or believing that one's pet was specifically at low risk. Cost was the least common reason for not administering preventive drugs. *Aedes albopictus* was the most abundant species in larval surveys of residential yards (88.2%) and *Culex quinquefasciatus* was second most abundant (11.7%). No mosquito pools tested positive for *D. immitis* (n=73). Community

knowledge and awareness could be improved by attention to areas identified as lacking, and veterinarians and public health officials could improve practices by emphasizing disease risk posed by peridomestic *Ae. albopictus* and *Culex quinquefasciatus*, the cost benefit of prevention versus treatment of dog heartworm, and detailed instructions for effective mosquito reduction and avoidance practices. This is the first study to perform community-level KAP surveys regarding dog heartworm, and is also the first to compare mosquito abundance and *D. immitis* infection rate with KAP responses.

INTRODUCTION

Dog heartworm disease, caused by the filarial worm *Dirofilaria immitis*, is a life-threatening disease of dogs, with over 47,000 reported cases across the US with detection in every state in 2012, and a high density of cases around the Mississippi Delta region and the Southeast (www.capcvet.org/parasite-prevalence-maps). Canids, predominantly domestic/feral dogs and coyotes, are the primary, definitive hosts of *D. immitis,* but domestic cats can develop serious complications due to heartworm-associated respiratory disease in response to infection with as few as one to three adult *D. immitis.* Mosquitoes are the only known vectors of the *D. immitis* parasite(Phillips 1939, Bowman and Atkins 2009). Despite modern veterinary treatments and macrocyclic lactone preventives available to pet owners, dog heartworm remains a major veterinary concern. Evidence for macrocyclic lactone resistance has necessitated recent protocol changes discontinuing the slow-kill treatment method for microfilaremic dogs (Bourguinat et al. 2011, Geary et al. 2011, Brown et al. 2012) in which monthly preventives are administered to reduce microfilaremia.

Other filarial diseases, such as onchocerciasis and lymphatic filariasis, have responded well to integrated control programs breaking the cycle of disease transmission with mass treatment of at-risk human populations, vector management, and reduction of vector-host contact(World Health Organization 2010, Cupp et al. 2011); however, *D. immitis* infects a broad range of mosquito species and can cycle between several species of definitive hosts. The growing list of putative dog heartworm vectors in the United States includes at least 25 mosquito species in the *Anopheles, Aedes, Culex,* and *Psorophora* genera, with this diversity reflected in their feeding patterns, choice of breeding habitat, and geographic range(Ledesma and Harrington 2011). Consequently, key vectors can vary region to region with local host and vector ecology.

Various biological and socio-behavioral components of heartworm risk have been proposed (Brown et al. 2012) including economic status, proximity to vector habitat (Sacks et al. 2003, 2004), vector species and strain susceptibility (Tiawsirisup 2007, Ledesma and Harrington 2011), and climate (Fortin and Slocombe 1981, Knight and Lok 1998); however, no comprehensive study of these relationships has been conducted in heartworm endemic communities. My study assessed resident and pet owner knowledge, attitudes, and practices regarding dog heartworm and mosquito vectors in relation to entomological surveys of individual residences and adult mosquito collections. I also sought to incriminate key heartworm vector species in each community by their blood feeding patterns and heartworm infection rate in field collected mosquitoes. At least 17 confirmed heartworm vector species are found in Florida (Kulasekera and Kramer 2001, Darsie and Ward 2005, Ledesma and Harrington

2011), and the broad species diversity of potential *D. immitis* vectors suggests that mammal biting mosquitoes are candidate vectors.

Study sites were selected using Florida county prevalence data in 2012, and included neighborhoods in one high prevalence county, Columbia County, and one low prevalence county, St. John's County.

Prominent heartworm vector candidates in my collection areas were *Aedes albopictus*, *Anopheles quadrimaculatus*, *Culex quinquefasciatus*, and *Psorophora ferox*. These species were caught in high numbers, feed on mammal blood to varying degrees, and carry infective stage *D. immitis* in natural populations (Darsie and Morris 2003, Ledesma and Harrington 2011).

My collection methods targeted resting and host-seeking female mosquitoes in residential communities of northern Florida. Species abundance, distribution, and heartworm infection status were analyzed. Below, I discuss the ecological implications for the study of *D. immitis* transmission and vector incrimination as well as the importance of these findings to dog heartworm disease control through better vector management practices and public health messaging.

Materials and Methods

General timing and sample sizes Lake City (Columbia County), FL and St. Augustine South (St. John's County), FL were chosen as study sites based on historical data for *D. immitis* prevalence in dogs (http://www.capcvet.org/parasite-prevalence-maps/). The annual heartworm prevalence in Columbia County is high (33.33% in 2011, 8.1% in 2012), but sample size is

consistently low in the CAPC dataset (n_{2011}= 6, n_{2012}=37). St. John's County dog heartworm prevalence is low (1.4% in 2011, 1.35% in 2012) based on a high number of reported test results (n_{2011}= 444,907; n_{2012}= 413,309). Knowledge, Attitudes and Practices (KAP) questionnaires and entomological surveys of natural and man-made container-breeding mosquitoes were administered to community residents from June 19th-July 24th, 2013. Pairs of research team members administered KAP and entomological surveys from 1000-1200 hrs, 1400-1800 hrs for a total of 46 and 50 questionnaires completed in St. Augustine South and Lake City, respectively.

Following KAP and container surveys, adult mosquitoes were collected from July 1st-July 26th from 0900-1200 hrs two days a week in each neighborhood from wooded areas and residences of consenting KAP survey participants.

KAP and entomological survey methods

Methods of Tuiten et al. (2009) were generally followed for KAP and entomological surveys. County mosquito control districts were notified of my study dates, affiliations, and my study goals to request residents' participation in questionnaires and entomological surveys of their property. Questionnaires were administered only to consenting adults, and participants were assured of the confidentiality of their responses. If a consenting adult was not present, researchers returned between 1700-1800 hrs for another attempt. A 10 min questionnaire was administered to participants by trained interviewers. KAP survey question responses are presented in Appendix D.

After the KAP survey, permission was obtained to conduct an entomological survey of their yard. Containers of standing water were counted, diameter and height were measured, and number of larvae was estimated and scored. Ground depressions, water in plants, and standing pools of water were included. Sub-samples of larvae and all pupae (when possible) were collected with a fish net, turkey baster, or pipette, and placed in labeled falcon tubes. Larvae were transported back to the laboratory and examined under a dissecting microscope to confirm species. Pupae were allowed to eclose for identification. Entomological survey data are shown in Table 4.4.

Mosquito collections

Adult mosquitoes were collected using large vegetation aspirators (Ponlawat and Harrington 2005), BG-Sentinel traps (http://www.bg-sentinel.com/), CO_2-baited CDC traps, and resting boxes (Edman et al. 1968). Collections were conducted each day between 0900-1200 hrs. Vegetation aspirations targeted brush, broad-leafed plants, bromeliads, and ground cover vegetation. Collection time and duration were recorded for each vegetation aspiration for calculation of yield per collection-hour.

Vegetation aspirators fitted with 1 gallon paint strainer bags (Master Craft, South El Monte, CA) were used to capture resting mosquitoes. Sample bags from each 10-20 min collection were immediately taped tightly to prevent mosquito escape. Collection bags and containers from all methods (resting boxes, vegetation aspirators, BG Sentinel traps, CO_2-baited CDC traps) were stored in a cooler on ice for transport back to the laboratory.

Mosquito processing and identification

Female mosquitoes were identified to species following published keys (Darsie and Morris 2003, Darsie and Ward 2005). Mosquitoes too damaged for morphological identification were identified by DNA barcoding according to methods described by Cywinska, Hunter, & Herbert (2006) with primers designed by Folmer et al. (1994). Blood fed mosquitoes were placed individually in sterile microcentrifuge tubes. Non-fed mosquitoes identified from the same collection were pooled by species and placed in aliquots of up to 20 mosquitoes per tube. All mosquitoes were stored at -20°C prior to DNA extraction.

Molecular detection of infective stage *D. immitis*

DNA extraction All DNA extractions were performed using a modified PureGene DNA extraction protocol (Gentra Systems, Minneapolis, MN). Mosquito heads and thoraces were screened for dog heartworm and used for DNA barcoding identification of damaged mosquito specimens; abdomens of blood fed mosquitoes at least half-full of blood were extracted individually for blood meal identification. Dissections of blood fed abdomens were performed with surgical blades flame-sterilized between each sample. Surgical blades were also flame-sterilized between dissections of non-fed mosquito pools. Each specimen was homogenized in 300 µl of cell lysis solution and incubated at 65°C for 15 min. Homogenization of head/thorax samples for *D. immitis* screening was performed with the addition of 20mg of 0.5mm diameter zirconia/silica beads (BioSpec Products, Inc., Bartlesville, OK). Lysate was chilled on ice for 2 min and 100 µl of protein precipitation solution was added. Solution was mixed by vortex for 20 secs before being centrifuged at 17900 x g for 5 min. Supernatant containing DNA was transferred to a tube containing chilled 100% isopropanol to produce a final solution of 70%

isopropanol. This was inverted several times to mix and held at -20°C for 10 min. Tubes were centrifuged at 17900 x g for 20 min. Supernatant was poured off and 500 µl of freshly made 70% ethanol solution was added to each tube containing the DNA pellet. Tubes were inverted by hand several times before centrifugation at 17900 x g for 20 min. Supernatant ethanol solution was carefully poured off before an additional 2 min of centrifugation at 17900 x g. Remaining ethanol was removed by pipette, and tubes were left to air-dry for 10 min. 50µl of dH_2O (Gibco) was added before storage at -20°C.

Screening mosquito pools for infective stage *D. immitis* DNA was extracted as described from pools of no more than 20 non-fed mosquito heads/thoraces. The following PCR assay screens mosquitoes for infective stage *D. immitis* using primers specific to a 203bp region of *D. immitis* cytochrome oxidase I (Rishniw et al. 2006). Each reaction contained 1.0 µl of sample template, 1.0 µl of 10 µM DI COI forward primer solution, 1.0 µl of 10 µM DI COI reverse primer solution, 0.75 µl of 50 mM $MgCl_2$, 0.5 µl of 10 mM dNTP, 5.0 µl of 5x GoTaq Flexi buffer (Promega), 0.15 µl of 5 U/µl GoTaq Flexi DNA polymerase (Promega), and 15.6 µl of dH_2O (Gibco). Reactions were cycled at 94°C for 2 min, 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, and 72°C for 7 min. Cycles 2-4 were repeated an additional 31 times. PCR products were separated on an ethidium bromide-stained 1% agarose gel in 1X TBE buffer for 60 mins at 120V before being visualized and digitally photographed using a Multi Doc-It Digital Imaging System (UVP, Inc.).

Prior testing with this method consistently detected single L3 stage *D. immitis* larvae in pools of up to 20 negative mosquito heads/thoraces. Two-fold serial dilutions of template DNA

from extractions of 10 L3 stage larvae yielded positive results with this assay with samples 1/32 of the original concentration, the equivalent of 5/16 of a single L3. Negative controls of non-spiked pools of mosquito heads/thoraces and positive controls of six L3 stage *D. immitis* larvae and 20 mosquito heads/thoraces spiked with one L3 were run with each test.

Target genes, sequences, names, and sources of all primers are presented in Appendix D.

Data analysis Factor independence from city, highest education, and dog ownership completed were tested by X^2 analyses of numbers in each answer category (df=1, $p<0.05$). Associations between demographics; knowledge, attitude, perceptions, and practices; and presence of mosquito larvae/pupae were analyzed using binary logistic regression. Presence of mosquitoes positive for *D. immitis* L3 stage larvae were also tested as an outcome variable of logistic binary regression. Quantitative measures of larvae/pupae found and *D. immitis* positive mosquitoes were analyzed as outcomes of separate linear regression models. Adult mosquito collection data were standardized by collection-hours and trap-nights before inclusion in models.

Resident responses were coded to ensure confidentiality.

RESULTS

KAP questionnaire

Lake City (Columbia County) (n=50) and St. Augustine South (St. John's County) (n=46) respondent demographics did not differ in education level or pet ownership, but US Census

Bureau records from 2012 indicate that St. Augustine residents' median income was about twice that of Lake City residents. The majority of respondents owned pets; 53.1% owned at least one dog and 30.2% percent owned at least one cat. Response totals less than 96 reflect either missing data, no answer for a question, or questions that were not applicable to all respondents. For those response categories that were not mutually exclusive sample sizes represent counts of answer selections. For some questions of knowledge or attitudes non-pet owners' answers reflect their decisions if they hypothetically owned a dog.

Mosquito-borne disease concern and knowledge varied greatly between diseases. Respondents were most familiar with West Nile virus and dog heartworm: 93.8% (WNV) and 98.0% (heartworm) of residents had some level of knowledge of these diseases. St. Augustine residents were more likely to be highly concerned about West Nile virus than Lake City residents (p=0.043). Most respondents (42.7%) had not heard of Eastern Equine Encephalitis (EEE, despite detection of the virus in sentinel animals in St. John's County before and during the survey period (http://diseasemaps.usgs.gov/). The majority of residents had not heard of Chikungunya (88.5%), St. Louis encephalitis (69.8%), or dengue (68.8%) (Table 4.1).

Lake City residents were more likely to be aware of the timing of mosquito activity during the day (p=0.015), and St. Augustine residents were more likely to perceive a high mosquito activity level on their property (p=0.013) (Table 4.1). Lake City residents were more likely to estimate treatment cost of heartworm infection to be under $1000 ($p$=0.028), and less likely to estimate treatment to be between $1000-2000 ($p$=0.011).

Residents who completed some college were more likely to be concerned about St. Louis Encephalitis virus than residents who completed high school or less (p=0.001) (Table 4.2). No other concern varied with education level (p<0.05). Residents who completed at least some college were more likely to know that dogs are at risk of heartworm disease than residents who completed high school or less (p=0.045) (Table 4.2).

Dog owners were more likely than non-dog owners to know that mosquitoes transmit dog heartworm (p<0.001), standing water is an important breeding site for mosquitoes (0.005), and they were also more likely (p=0.036) to perceive high mosquito activity levels on their property (Table 4.3). Non-dog owners were more likely to know that mosquitoes are active at dawn (0.039).

Dog owners were less likely (p=0.007) to state "No concern" and more likely (p=0.03) to state "High concern" regarding dog heartworm than residents who did not own dogs (Table 4.3). Dog owners were also more likely (p=0.009) to estimate treatment of dog heartworm would cost between $1000-2000, while non-dog owners were more likely (p=0.011) than dog owners to estimate treatment costs below $1000. Dog owners were more likely to be willing to pay over $1500 for treatment ($p$=0.018) than non-dog owners, and non-dog owners were more likely (p=0.011) to only be willing to pay up to $100 for treatment if they were to own a dog.

The majority of pet owners (71.2%) had their dogs on preventive drugs. Only one had a cat on preventive. Pet owners not administering preventive (n=19) provided their reasons (Figure 4.1), the top three of which were:

1. They did not believe their pet was at risk

2. They had never considered that their pet could be infected
3. They do not know why they do not administer preventive

Cost was the least common reason for not administering preventive.

Table 4.1: Demographics, knowledge, attitudes, and practices comparisons between St. Augustine South and Lake City. Responses with significant differences between neighborhoods as determined by chi-squared analysis of each response (df=1, p <0.05) are shaded and in **bold.**

		St. Augustine n (%)	Lake City n (%)	p value
Highest education completed	High School or lower	10 (22.2)	20 (40.8)	0.053
	Some college or more	35 (77.8)	29 (59.2)	
Median yearly income		$61, 288	$36, 542	
Pets owned	None	9 (19.6)	17 (34.0)	0.112
	Dogs only	18 (39.1)	18 (36.0)	0.752
	Cats only	5 (10.9)	9 (18.0)	0.323
	Dog/s and cat/s	10 (21.7)	5 (10.0)	0.114
	Other	4 (8.7)	1 (2.0)	0.140
WNV concern	None	6 (13.0)	13 (26.0)	0.111
	Low	6 (13.0)	5 (10.0)	0.640
	Moderate	5 (10.9)	9 (18.0)	0.323
	High	**27(58.7)**	**19 (38.0)**	**0.043**
	Don't know/Can't rate	2 (4.3)	4 (8.0)	0.460
DEN concern	None	7 (15.2)	7 (14.0)	0.866
	Low	3 (6.5)	1 (2.0)	0.268
	Moderate	2 (4.3)	1 (2.0)	0.509
	High	5 (10.9)	4 (8.0)	0.630
	Don't know/Can't rate	29 (63.0)	37 (74.0)	0.247
Heartworm concern	None	8 (17.4)	9 (18.0)	0.938
	Low	3 (6.5)	1 (2.0)	0.268
	Moderate	7 (15.2)	4 (8.0)	0.267
	High	27 (58.7)	35 (70.0)	0.247
	Don't know/Can't rate	1 (2.2)	1 (2.0)	0.952
EEE concern	None	8 (17.4)	9 (18.0)	0.938
	Low	8 (17.4)	3 (6.0)	0.080
	Moderate	6 (13.0)	5 (10.0)	0.640
	High	8 (17.4)	9 (18.0)	0.938
	Don't know/Can't rate	16 (34.8)	24 (48.0)	0.189

SLE concern	None	5 (10.9)	6 (12.0)	0.862
	Low	5 (10.9)	2 (4.0)	0.196
	Moderate	2 (4.3)	2 (4.0)	0.932
	High	3 (6.5)	4 (8.0)	0.781
	Don't know/Can't rate	31 (67.4)	36 (72.0)	0.623
CHK concern	None	2 (4.3)	3 (6.0)	0.716
	Low	0 (0)	1 (2.0)	0.335
	Moderate	1 (2.2)	1 (2.0)	0.952
	High	0 (0)	3 (6.0)	0.091
	Don't know/Can't rate	43 (93.5)	42 (84.0)	0.145
Hosts	Dogs can be infected	34 (56.7)	36 (58.1)	0.914
	Cats can be infected	23 (38.3)	21 (33.9)	0.522
	Don't know	3 (5.0)	5 (8.1)	
Transmission	Mosquitoes	23 (50.0)	16 (32.0)	0.073
	Mosquitoes not mentioned	23 (50.0)	34 (68.0)	
Seriousness of heartworm infection	Moderately serious	1 (2.4)	1 (2.3)	0.959
	Highly serious	39 (95.1)	42 (97.7)	0.959
	Don't know	1 (2.4)	0 (0)	
Daily mosquito activity	Dawn	18 (39.1)	18 (36.0)	0.752
	Day	**0 (0)**	**6 (12.0)**	**0.015**
	Dusk	38 (82.6)	39 (78.0)	0.571
	Night	8 (17.4)	14 (28.0)	0.217
Mosquito breeding sites	Standing water	39 (84.8)	37 (74.0)	0.194
	Standing water not mentioned	7 (15.2)	13 (26.0)	
Frequency of eliminating standing water	Once a week or more	28 (62.2)	23 (46.9)	0.137
	Never	11 (24.4)	16 (32.7)	0.380
	Once a month	4 (8.9)	2 (4.1)	0.341
	Don't know	2 (4.4)	8 (16.3)	0.062
Highest cost willing to pay	Less than $100/not willing	2 (4.9)	2 (4.7)	0.961

	Up to $100	8 (19.5)	9 (20.9)	0.872
	Up to $500	13 (31.7)	15 (34.90	0.758
	Up to $1000	4 (9.8)	3 (7.0)	0.645
	Over $1500	14 (34.1)	14 (32.6)	0.877
Perceived level of mosquito activity	None	1 (2.2)	2 (4.1)	0.608
	Low	13 (28.9)	16 (32.7)	0.693
	Medium	11 (24.4)	21 (42.9)	0.060
	High	**20 (44.4)**	**10 (20.4)**	**0.013**
Frequency of vet visits: dog owners	Once a year	11 (40.7)	10 (43.5)	0.695
	More than once a year	12 (44.4)	10 (43.5)	0.501
	Only when necessary/Less than once a year	3 (11.1)	3 (13.0)	0.873
	Don't know	1 (3.7)	0 (0)	
Frequency of vet visits: cat owners	Once a year	7 (46.7)	4 (28.6)	0.316
	More than once a year	4 (26.7)	3 (21.4)	0.742
	Only when necessary/Less than once a year	4 (26.7)	4 (28.6)	0.909
	Never	0 (0)	3 (21.4)	0.058
Has vet informed clients about heartworm?	Yes	30 (90.9)	26 (78.8)	0.170
	No	3 (9.1)	7 (21.2)	
Preventive administration	All year	26 (96.3)	15 (79.0)	0.063
	Less	1 (3.7)	4 (21.1)	
Prevention status	Not on preventive	8 (22.9)	11 (35.5)	0.258
	On preventive	27 (77.1)	20 (64.5)	
Estimated cost of treatment	**Less than $1000**	**14 (34.1)**	**25 (58.1)**	**0.028**
	$1000-2000	**13 (31.7)**	**4 (9.3)**	**0.011**
	Over $2000	3 (7.3)	1 (2.3)	0.283
	Other	2 (4.9)	2 (4.7)	0.961
	Don't know	9 (78)	11 (25.6)	0.696

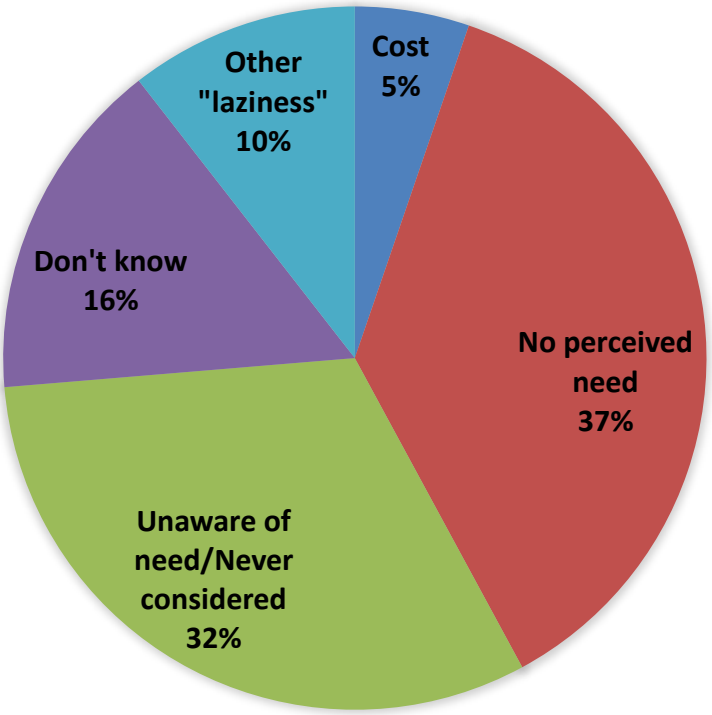
Table 4.2: Knowledge and attitudes comparisons between education levels. Responses with significant differences between education levels as determined by chi-squared analysis of each response (df=1, p <0.05) are shaded and in **bold.**

		High school or less n (%)	At least some college n (%)	p value
SLE concern	**No concern**	**6 (20.0)**	**4 (6.3)**	**0.001**
	Low concern	1 (3.3)	6 (9.4)	0.606
	Moderate concern	1 (3.3)	3 (4.7)	0.130
	High concern	4 (13.3)	3 (4.7)	0.334
	Don't know,can't rate	18 (60.0)	48 (75.0)	0.646
Dogs as hosts	**Dogs not mentioned**	**9 (30.0)**	**8 (12.5)**	**0.045**
	Dogs are hosts	**19 (63.3)**	**50 (78.1)**	**0.045**
	Don't know	2 (6.7)	6 (9.4)	0.632

Table 4.3: Demographics, knowledge and attitudes comparisons between dog owners and non-dog owners. Responses with significant differences between dog ownership as determined by chi-squared analysis of each response (df=1, p <0.05) are shaded and in **bold.**

		Do not own dogs n (%)	Dog owners n (%)	p value
Education level	High school or less	9 (20)	14 (28.6)	0.334
	At least some college	36 (80)	35 (71.4)	
Heartworm concern	**None**	**13 (28.9)**	**4 (7.8)**	**0.007**
	Low	1 (2.2)	3 (5.9)	0.37
	Moderate	5 (11.1)	6 (11.8)	0.92
	High	**24 (53.3)**	**38 (74.5)**	**0.03**
	Don't know,can't rate	2 (4.4)	0 (0)	0.128
Are dogs heartworm hosts?	Dogs are not hosts	9 (20)	9 (17.6)	0.768
	Dogs are hosts	30 (66.7)	40 (78.4)	0.196
	Don't know	6 (13.3)	2 (3.9)	0.096
Heartworm transmission	**Not transmitted by mosquitoes**	**37 (82.2)**	**20 (39.2)**	**<0.001**
	Mosquito transmission	**8 (17.8)**	**31 (60.8)**	**<0.001**
Estimated cost of treatment	**Under $1000**	**21 (63.6)**	**18 (35.3)**	**0.011**
	$1000-2000	**2 (6.1)**	**15 (29.4)**	**0.009**
	Over $2000	1 (3.0)	3 (5.9)	0.549
	Other/Don't know	9 (27.3)	15 (29.4)	0.832
Most willing to pay for treatment	**Less than $100/not willing**	**4 (12.1)**	**0 (0)**	**0.011**
	Up to $100	8 (24.2)	9 (17.6)	0.462
	Up to $500	13 (39.4)	15 (29.4)	0.343
	Up to $1000	2 (6.1)	5 (9.8)	0.544
	Over 1500	**6 (18.2)**	**22 (43.1)**	**0.018**
Perceived mosquito activity	None	2 (4.7)	1 (2.0)	0.460
	Low	13 (30.2)	16 (31.4)	0.905
	Medium	19 (44.2)	13 (25.5)	0.057
	High	**9 (20.9)**	**21 (41.2)**	**0.036**
Daily mosquito activity	**Dawn**	**33 (73.3)**	**27 (52.9)**	**0.039**
	Day	3 (6.7)	3 (5.9)	0.874
	Dusk	35 (77.8)	42 (82.4)	0.574

	Night	10 (22.2)	12 (23.5)	0.879
Mosquito breeding sites	**Standing water mentioned**	**30 (66.7)**	**46 (90.2)**	**0.005**
	Standing water not mentioned	**15 (33.3)**	**5 (9.8)**	**0.005**



n=19

Figure 4.1: Pet owner reasons for not administering preventive.

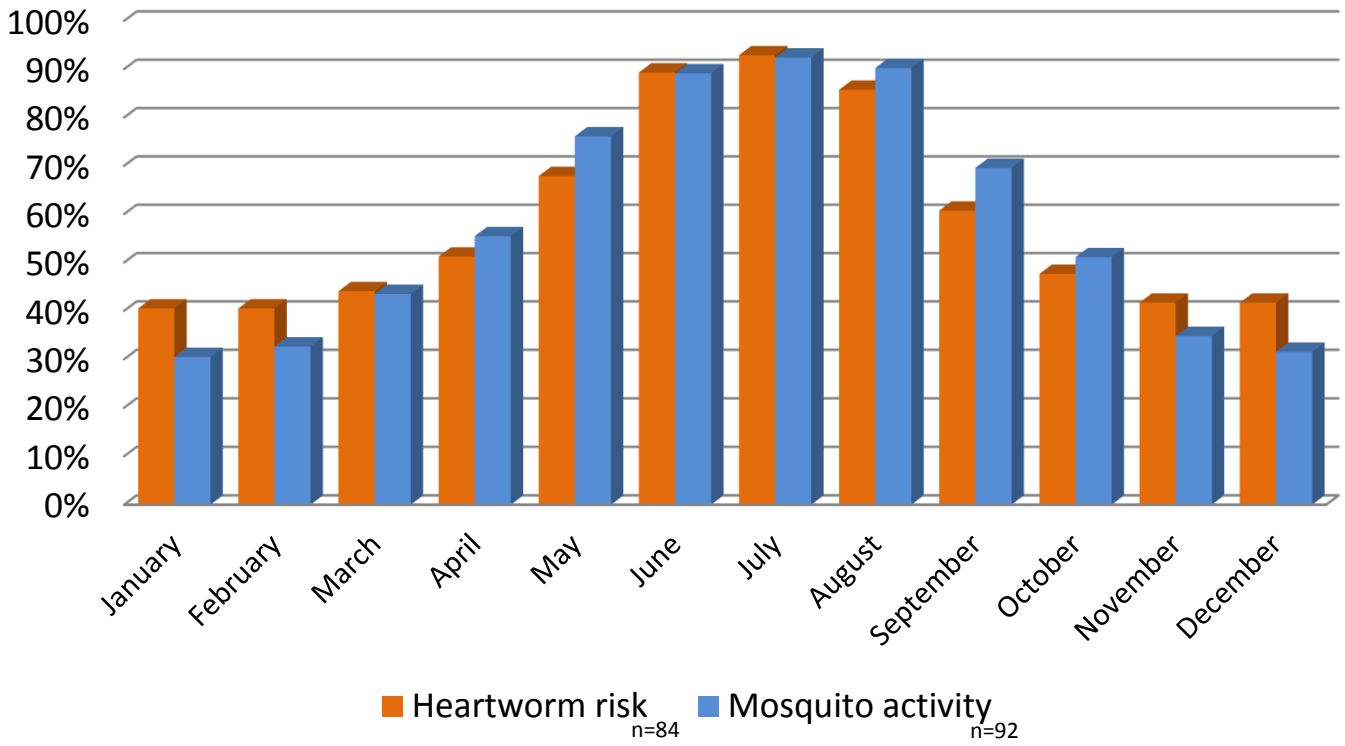


Figure 4.2: Perceived heartworm risk and mosquito activity per month.

Residents were asked to name the months during which animals are at risk of being infected with heartworm and, in a different part of the questionnaire, they were also asked to name months during which mosquitoes are active. These responses followed a similar distribution: over 80% of responses included June, July, and August; the least frequently mentioned months were November-February; and over 50% of heartworm risk responses spanned April-September, while over 50% of mosquito activity responses spanned April-October (Figure 4.2).

Entomological surveys

Aedes albopictus was the most abundant mosquito (88.2%) in container surveys and *Culex quinquefasciatus* was the next most abundant species (11.7%) (Table 4.4). Since *Toxorhynchites r. septentrionalis* (0.0012%) do not feed on blood, *Ae. albopictus* and *Cx. quinquefasciatus* were the only potential vector species breeding in standing water and artificial containers on residents' property.

Table 4.4: Entomological survey indices, larval abundance, and distribution.

[a]Percentage of positive containers

[b]Percentage of houses with positive containers

	St. Augustine	Lake City
Total containers	140	147
Total houses	52	52
Container index[a]	56%	37%
House index[b]	65%	48%
Total larvae	351	472
Ae. albopictus	292 (83.2%)	434 (91.9%)
Cx. quinquefasciatus	59 (16.8)	37 (7.8%)
Toxorhynchites r. septentrionalis	0 (0%)	1 (0.2%)

Table 4.5: Trap-nights (CDC, BG, RB) or collection-hours (VC) by location.

	St. Augustine	Lake City
CDC	31	24
BG	28	24
RB	32	20
VC	6:50	3:30

CDC: CO_2-baited CDC trap

BG: BG sentinel trap

RB: Resting box

VC: Large vegetation aspirator

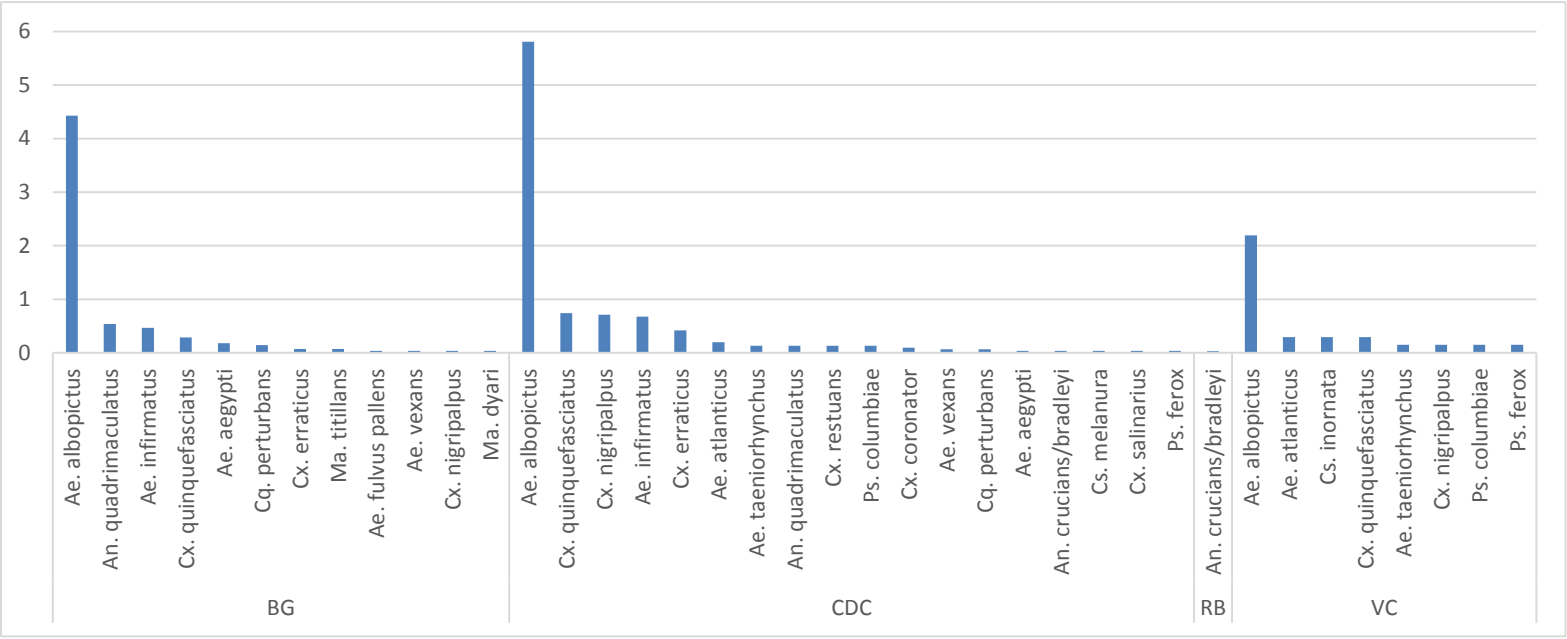


Figure 4.3: Mosquito species by method and collection effort in St. Augustine. Total St. Augustine catch (n=496) is presented in terms of mosquito species per collection-hours (VC) or trap-nights (BG, CDC, RB).

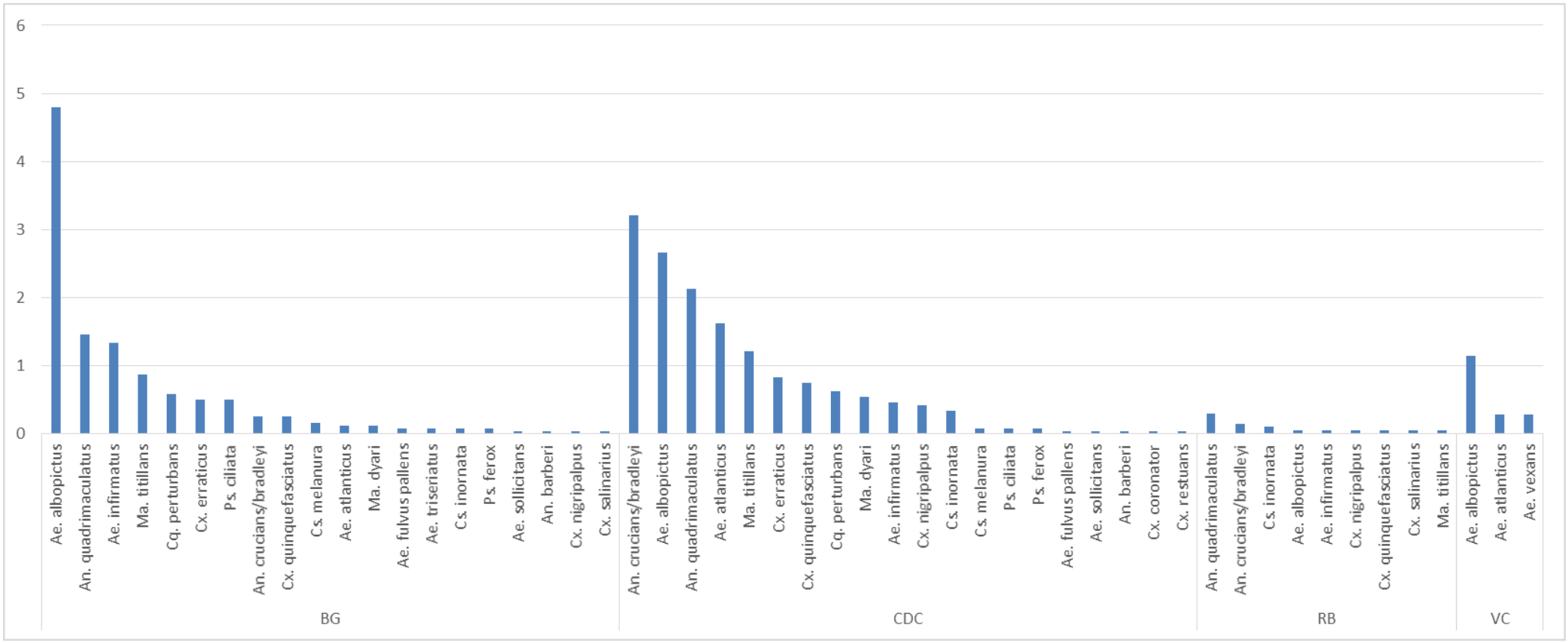


Figure 4.4: Mosquito species by method and collection effort in Lake City. Total Lake City catch (n=665) is presented in terms of mosquito species per collection-hours (VC) or trap-nights (BG, CDC, RB).

Adult mosquito collections

BG Sentinel trap, CDC trap, and resting box collection effort were similar between cities, although I accumulated about half the aspiration collection-hours in Lake City than in St. Augustine (Table 4.5). *Aedes albopictus* represented the majority of my collection, and BG Sentinel traps, CDC traps, and vegetation aspirations were the most effective means of collecting them (Figures 4.3 and 4.4). *Anopheles crucians* and *An. quadrimaculatus* were caught most efficiently by CDC and BG trapping, and were almost absent from St. Augustine collections. Although species represented by both collections were similar—23 species in Lake City and 22 in St. Augustine—abundance across species collected per effort was more evenly distributed in Lake City, and *Ae. albopictus* represented a smaller proportion of total catch than in St. Augustine.

***D. immitis* screening of mosquito pools**

Of 166 pools of mosquito heads/thoraces tested, none were positive for *D. immitis* (Table 4.6).

Table 4.6: Mosquito pools of heads/thoraces screened for *D. immitis.* Location, collection date, species, and number of mosquitoes per pool are shown. LC=Lake City, SA=St. Augustine

City	Date	Species	#	City	Date	Species	#	City	Date	Species	#
SA	07/02/13	*Ae. infirmatus*	9	LC	07/18/13	*Ma. titillans*	1	LC	07/19/13	*Ma. titillans*	2
SA	07/02/13	*An. quadrimaculatus*	1	LC	07/18/13	*Ma. titillans*	1	LC	07/19/13	*Ma. titillans*	1
SA	07/03/13	*Aedes sp.*	1	LC	07/18/13	*Ma. titillans*	1	LC	07/19/13	*Ma. titillans*	1
SA	07/03/13	*Cx. erraticus*	2	LC	07/18/13	*Ma. titillans*	1	LC	07/19/13	*Ma. titillans*	1
SA	07/03/13	*Cx. quinquefasciatus*	1	LC	07/18/13	*Ps. ciliata*	1	LC	07/19/13	*Ps. ciliata*	1
SA	07/09/13	*Ae. albopictus*	1	LC	07/18/13	*Ps. ciliata*	1	LC	07/19/13	*Ps. ciliata*	1
SA	07/09/13	*Ae. albopictus*	1	SA	07/18/13	*Ae. atlanticus*	1	LC	07/19/13	*Ps. ciliata*	1
SA	07/09/13	*Cx. erraticus*	1	LC	07/19/13	*Ae. albopictus*	6	LC	07/19/13	*Ps. ferox*	1
SA	07/09/13	*Cx. salinarius*	1	LC	07/19/13	*Ae. albopictus*	5	LC	07/19/13	*Ps. ferox*	1
SA	07/10/13	*Ae. albopictus*	11	LC	07/19/13	*Ae. albopictus*	3	SA	07/19/13	*An. bradleyi/crucians*	2
SA	07/10/13	*Ae. albopictus*	1	LC	07/19/13	*Ae. albopictus*	3	SA	07/23/13	*Ae. albopictus*	1
SA	07/10/13	*Cx. nigripalpus*	1	LC	07/19/13	*Ae. albopictus*	2	SA	07/23/13	*Ae. albopictus*	1
SA	07/10/13	*Cx. quinquefasciatus*	1	LC	07/19/13	*Ae. albopictus*	1	SA	07/23/13	*Ae. atlanticus*	1
SA	07/14/13	*Ae. albopictus*	1	LC	07/19/13	*Ae. albopictus*	1	SA	07/23/13	*Cs. inornata*	2
SA	07/14/13	*Cx. quinquefasciatus*	5	LC	07/19/13	*Ae. albopictus*	1	SA	07/23/13	*Ps. columbiae*	1
LC	07/15/13	*An. quadrimaculatus*	1	LC	07/19/13	*Ae. atlanticus*	8	SA	07/23/13	*Ps. ferox*	1
LC	07/15/13	*Ma. titillans*	2	LC	07/19/13	*Ae. atlanticus*	1	SA	07/24/13	*Ae. albopictus*	11
SA	07/15/13	*An. quadrimaculatus*	2	LC	07/19/13	*Ae. fulvus pallens*	2	SA	07/24/13	*Ae. albopictus*	6
SA	07/16/13	*Ae. albopictus*	21	LC	07/19/13	*Ae. infirmatus*	4	SA	07/24/13	*Ae. albopictus*	5
SA	07/16/13	*Ae. albopictus*	8	LC	07/19/13	*Ae. infirmatus*	1	SA	07/24/13	*Ae. albopictus*	3
SA	07/16/13	*Ae. albopictus*	3	LC	07/19/13	*Ae. triseriatus*	2	SA	07/24/13	*Ae. atlanticus*	4
SA	07/16/13	*Ae. albopictus*	2	LC	07/19/13	*Aedes sp.*	1	SA	07/24/13	*Ae. atlanticus*	1
SA	07/16/13	*Ae. albopictus*	2	LC	07/19/13	*An. bradleyi/crucians*	3	SA	07/24/13	*Ae. fulvus pallens*	1
SA	07/16/13	*Ae. taeniorhynchus*	1	LC	07/19/13	*An. bradleyi/crucians*	2	SA	07/24/13	*Ae. infirmatus*	6
SA	07/16/13	*Ae. taeniorhynchus*	1	LC	07/19/13	*An. bradleyi/crucians*	1	SA	07/24/13	*Ae. infirmatus*	2
LC	07/17/13	*Ae. atlanticus*	4	LC	07/19/13	*An. bradleyi/crucians*	1	SA	07/24/13	*Ae. infirmatus*	1
SA	07/17/13	*Ae. albopictus*	8	LC	07/19/13	*An. bradleyi/crucians*	1	SA	07/24/13	*An. bradleyi/crucians*	1

SA	07/17/13	*Ae. infirmatus*	1	LC	07/19/13	*An. quadrimaculatus*	9	SA	07/24/13	*An. quadrimaculatus*	16
SA	07/17/13	*Cx. erraticus*	1	LC	07/19/13	*An. quadrimaculatus*	3	SA	07/24/13	*Cq. perturbans*	2
SA	07/17/13	*Cx. salinarius*	1	LC	07/19/13	*An. quadrimaculatus*	3	SA	07/24/13	*Cq. perturbans*	1
LC	07/18/13	*Ae. albopictus*	6	LC	07/19/13	*An. quadrimaculatus*	2	SA	07/24/13	*Cx. coronator*	1
LC	07/18/13	*Ae. albopictus*	4	LC	07/19/13	*An. quadrimaculatus*	2	SA	07/24/13	*Cx. quinquefasciatus*	1
LC	07/18/13	*Ae. albopictus*	2	LC	07/19/13	*An. quadrimaculatus*	1	SA	07/24/13	*Ma. dyari*	1
LC	07/18/13	*Ae. albopictus*	2	LC	07/19/13	*An. quadrimaculatus*	1	SA	07/24/13	*Ma. titillans*	1
LC	07/18/13	*Ae. albopictus*	1	LC	07/19/13	*An.bradleyi/crucians*	2	SA	07/24/13	*Ma. titillans*	1
LC	07/18/13	*Ae. atlanticus*	2	LC	07/19/13	*Cq. perturbans*	2	SA	07/24/13	*Ps. ferox*	1
LC	07/18/13	*Ae. atlanticus*	1	LC	07/19/13	*Cq. perturbans*	2	LC	07/25/13	*Ae. albopictus*	13
LC	07/18/13	*Ae. atlanticus*	1	LC	07/19/13	*Cq. perturbans*	1	LC	07/25/13	*Ae. infirmatus*	2
LC	07/18/13	*Ae. infirmatus*	17	LC	07/19/13	*Cq. perturbans*	1	LC	07/25/13	*Ae. infirmatus*	1
LC	07/18/13	*Ae. infirmatus*	1	LC	07/19/13	*Cq. perturbans*	1	LC	07/25/13	*An. bradleyi/crucians*	2
LC	07/18/13	*Ae. infirmatus*	1	LC	07/19/13	*Cq. perturbans*	1	LC	07/25/13	*Anopheles sp.*	1
LC	07/18/13	*Ae. infirmatus*	1	LC	07/19/13	*Cs. inornata*	2	LC	07/25/13	*Cx. erraticus*	1
LC	07/18/13	*An. bradleyi/crucians*	4	LC	07/19/13	*Cs. melanura*	1	LC	07/25/13	*Cx. quinquefasciatus*	1
LC	07/18/13	*An. bradleyi/crucians*	2	LC	07/19/13	*Cx. erraticus*	1	LC	07/25/13	*Ma. titillans*	4
LC	07/18/13	*An. bradleyi/crucians*	1	LC	07/19/13	*Cx. erraticus*	1	LC	07/25/13	*Ma. titillans*	2
LC	07/18/13	*An. punctipennis*	3	LC	07/19/13	*Cx. nigripalpus*	3	LC	07/26/13	*Ae. albopictus*	8
LC	07/18/13	*An. quadrimaculatus*	2	LC	07/19/13	*Cx. nigripalpus*	1	LC	07/26/13	*Ae. albopictus*	1
LC	07/18/13	*An. quadrimaculatus*	2	LC	07/19/13	*Cx. quinquefasciatus*	3	LC	07/26/13	*Ae. infirmatus*	1
LC	07/18/13	*An. quadrimaculatus*	1	LC	07/19/13	*Cx. quinquefasciatus*	1	LC	07/26/13	*An. quadrimaculatus*	4
LC	07/18/13	*An. quadrimaculatus*	1	LC	07/19/13	*Cx. quinquefasciatus*	1	LC	07/26/13	*An. quadrimaculatus*	3
LC	07/18/13	*An. quadrimaculatus*	1	LC	07/19/13	*Ma. dyari*	2	LC	07/26/13	*Cq. perturbans*	2
LC	07/18/13	*Cq. perturbans*	2	LC	07/19/13	*Ma. dyari*	2	LC	07/26/13	*Cs. melanura*	1
LC	07/18/13	*Cq. perturbans*	1	LC	07/19/13	*Ma. dyari*	1	LC	07/26/13	*Cx. erraticus*	1
LC	07/18/13	*Cx. erraticus*	5	LC	07/19/13	*Ma. titillans*	3	LC	07/26/13	*Cx. quinquefasciatus*	8
LC	07/18/13	*Cx. nigripalpus*	1	LC	07/19/13	*Ma. titillans*	2	LC	07/26/13	*Ma. titillans*	3

LC	07/28/13	*An. quadrimaculatus*	2

DISCUSSION

My study revealed associations between knowledge, perception, and prevention practices relevant to mosquito-borne disease and, specifically, dog heartworm. Although widespread moderate to high concern about dog heartworm could be a product of my disclosure of the subject and purpose of my questionnaire, lack of awareness and concern for dengue and EEE is interesting since human cases of both were detected in Florida that transmission season, and both counties have active mosquito control districts. Residents were aware of SLE and concern varied with education level, but this may also reflect residents' familiarity with the term "encephalitis."

Residents' understanding of dog heartworm transmission biology and risk was often incorrect. When asked to list months during which animals were at risk of contracting heartworm and, in a separate question, when mosquitoes are active during the year, only 40.5% of resident response distribution covered the whole year for heartworm risk, and 30.4% covered all year mosquito activity. The two similar response distributions suggest that residents connected mosquito activity to heartworm risk, however, 59.4% of residents did not know that mosquitoes transmit dog heartworm (Table 4.1). Knowing that dogs can be hosts of dog heartworm was associated with education level but not dog ownership (Tables 4.2 and 4.3), and only 57.4% of resident responses named dogs as potential heartworm hosts (Table 4.1). Most residents also believed that daily mosquito activity was restricted to dawn and dusk despite my entomological surveys and adult mosquito collections (Table 4.1, Figures 4.3 and

4.4), which included large numbers of *Ae. albopictus*(diurnal feederl) and *Cx. quinquefasciatus*(nocturnal feeder).

Most pet owners had their pets on preventive (Table 4.1), and only one of these pets was a cat. For the first time, reasons for non-compliance with recommendations to administer preventive macrocyclic lactone are quantified and, unexpectedly, cost was the least common reason for non-administration (Figure 4.1). Lack of risk awareness and individual perception of low risk were the top two reasons for non-compliance. The uninformed status of certain pet owners could be related to the frequency with which they take their pet/s to the veterinary visits, which itself could be an indicator of economic status, attitude towards pet ownership, or other social factors not analyzed in this study. Correct estimation of the cost of veterinary treatment for heartworm infection was associated with living in St. Augustine and with dog ownership, which may reflect socioeconomic factors allowing St. Augustine residents to have more experience with veterinary costs, and dog owners tended to provide a higher frequency of veterinary visits for their pets (Tables 4.1 and 4.3).

Although many potential risk factors and topics requiring more messaging attention are indicated by my study, the primary limitations to my conclusions are that participants were not chosen at random, many personal demographic factors were not studied, and only two neighborhoods were surveyed over a single six-week period. KAP interviewers were also required to disclose dog heartworm as the topic of my study, which could have artificially increased the level of concern and importance reflected in responses regarding dog heartworm. Knowledge of dog heartworm, however, should not have been affected. Since questionnaires

were administered in-person, responses may have been skewed in cases where participants may have been embarrassed or unwilling to disclose information, such as mentioning cost as their reason for not administering preventive drugs. I designed my study to capture mosquitoes during peak summer transmission period, but adult mosquito collections only spanned a four-week period.

This is the first community-scale KAP study of dog heartworm, and it is also the first to relate mosquito abundance, infection rate, and peridomestic breeding sites to KAP study findings. My study sites within St. John's County (St. Augustine South) and Columbia County (Lake City) were chosen to compare owner sociobehavioral factors between known prevalence rates, ecological factors, and demographics. Both counties have active mosquito control districts that provide mosquito reduction services and alert residents to public health threats of mosquito vectors. My approach may provide a framework for integrated heartworm vector control strategies to target key vectors in regions of interest. *D. immitis* screening, categorization of breeding sites, and species distribution can be synthesized to pinpoint key vector species likely to be involved in maintaining *D. immitis* in the population. Continued surveillance in this manner could help mosquito control programs monitor and report the spread of *D. immitis* similar to surveillance programs in place for reporting cases of West Nile Virus, Eastern Equine Encephalitis Virus, and Dengue Virus (www.diseasemaps.usgs.gov).

Although I cannot infer a causal relationship between significant associations in my KAP questionnaire findings, implications remain for public health messaging from veterinarians and mosquito control districts to the communities they serve. Emphasis on the cost of owning and

providing care for dogs and cats could motivate pet owners to administer preventive drugs to their dogs and cats, and veterinarians could strengthen their relationships with their clients by presenting the potential high expenses associated with hospitalization and treatment of heartworm disease in comparison to the relatively low cost of monthly preventives and veterinary check-ups. Most dog owners estimated the cost of treatment for dog heartworm infection to be less than $1000, which would be unlikely given collateral expenses on hospitalization fees and supportive care. Although cost was the least common reason for not administering preventive drugs to pets, this could be due to an unrealistic calculation between cost of monthly preventive drugs and residents' low estimation of veterinary fees. Misconceptions leading residents to believe that their animals are at low risk should also be addressed as ignorance of risk and personal perception of low risk were the top two reasons for non-administration of preventive drugs. It should be emphasized that cats and indoor dogs are at risk of *D. immitis* infection and regional dog or vector prevalence data may help pet owners make informed decisions regarding their pets' health.

Public messaging regarding mosquito reduction practices and their importance as disease vectors may improve resident knowledge and awareness of disease risk. Although most residents knew that standing water was an important breeding site for mosquitoes, positive container indices between communities and houses were similar (Table 4.4) and many residents who claimed that they did not eliminate standing water because it was not found on their property did not realize that artificial containers in garden ornaments, plant pots, and children's toys are potential mosquito breeding sites. Upon surveying yards for mosquito larvae, I also learned that many residents did not know what mosquito larvae look like.

Mosquito control district messaging may benefit from including pictures of mosquito larvae and garden objects that could serve as mosquito breeding containers in their communications to their communities.

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APPENDIX A: DNA barcoding troubleshooting and optimization

Goals

To develop an unbiased method for mosquito blood meal analysis of host origin using DNA barcoding. This method must:

- identify a wide-range of mammal, avian, and herpetological blood meal hosts
- be able to detect small quantities of partially digested DNA present in mosquito midgets
- selectively amplify vertebrate template over mosquito and microbe template

Obstacles

Non-barcoding methods of blood meal analysis can produce erroneous results, especially when determining avian from mammalian blood meals. Most do not offer strategies for identification of amphibian and reptile DNA. DNA barcoding was chosen for its ability to target unknown vertebrate hosts, specificity of COI sequence polymorphisms with which to make species-level identifications, and ability to process hundreds of samples with ease. Optimization occurred at several steps of the process:

- Primer selection/design
    - Initially many primers were identified as candidates for DNA barcoding blood meal analysis, and their method of design and target sequences were compared. Primers designed by alignment to specific target animals were discarded from consideration, and I favored primers that aimed to be as general as possible while still distinguishing vertebrate sequence from mosquito. I also gave more consideration to primers used in studies with similar host species presence as my study sites.

- Nested/semi-nested
    - Townzen et al. (2008) suggest that COI long F and short R primers can be used together to amplify PCR product of samples that produced weak signal with the Long set of primers. Trials of this method improved amplification of some samples, but sequence length was short and semi-nested product quality was poor. A nested amplification of long primer product with short primers produced small or no improvement over initial amplification.
- Annealing temperature
    - To increase amplification for low-quality or small samples, I sought to test a gradient of temperatures starting with the lowest annealing temperature of barcoding primer sets to the highest. Positive controls were chosen from samples that had previously amplified and sequenced well and other samples that had amplified and sequenced poorly. Temperature gradients could be tested by manually testing different PCR cycle programs or, more efficiently, with automated temperature gradient settings available on certain thermocyclers. Optimal annealing temperature of 50°C was determined for COI long and COI short primers by amplification success rate as observed with SDS-PAGE.
- $MgCl_2$ concentration
    - DNA barcoding using degenerate primers was observed to be sensitive to $MgCl_2$ concentrations. Once a primer set and protocol were chosen,

$MgCl_2$ concentrations were tested in 0.5mM increments above and below the original recommendation. Amplification of known and wild-caught samples was visually assessed by SDS-PAGE and the concentration producing the brightest and cleanest band at the target length was chosen. MgCl2-free buffer was chosen to avoid variation in premixed buffers.

- Blood meal size
    - The amount of DNA available for analysis varies with size of the blood meal, which can also indicate state of degradation from digestion. To maximize the efficiency of my methods and use of materials, I visually assessed the size of each mosquito blood meal as a proportion of its full volume. I initially analyzed all blood meals and some gravid mosquitoes to verify my methods, and found that half-sized blood meals were the lowest volume yielding good amplification and match success rates.

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APPENDIX B: Troubleshooting *Dirofilaria immitis* rearing and harvesting

Goals

Methods were optimized to:

- infect *Ae. aegypti* Liverpool strain mosquitoes with *D. immitis* microfilariae
- harvest and store up to 600 infective stage *D. immitis* within one day of harvesting

Obstacles

Infections of my *Ae. aegypti* Liverpool strain and *Ae. albopictus* New Jersey strain incubated at 28°C for 14-16 days—standard conditions for mass rearing of *D. immitis* (personal communication: Andy Moorhead, Christopher Evans, University of Georgia; Michael Ulrich, Cheri-Hill Kennel & Supply Inc. Stanwood, MI)—resulted in high mortality and no infective stages in either mosquito species. Migrated microfilariae in Malpighian tubules did not grow or molt (Figure B1).

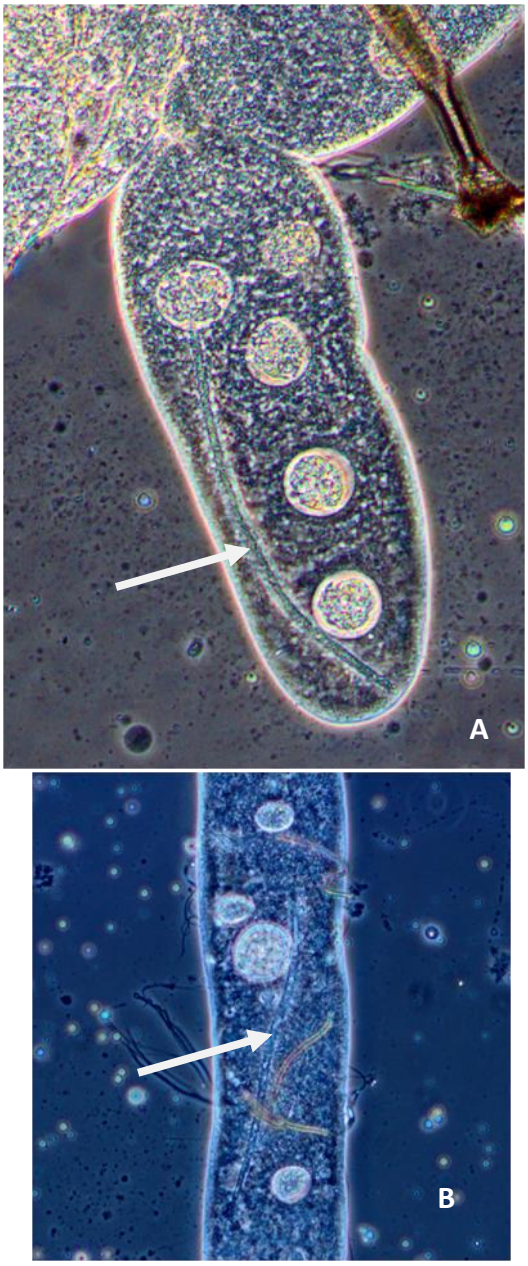

Figure B1: *Dirofilaria immitis*(white arrows) in arrested development in *Ae. aegypti* Malpighian tubules 10 days post-infection at 28°C (A and B). 200X

Approach

Causes of mosquito mortality and *D. immitis* arrested development were identified:

- Dehydration: Since *D. immitis* larvae develop and damage mosquito Malpighian tubules, I hypothesized that *D. immitis* development impedes osmoregulation in infected mosquitoes. Trays and other wide, open containers were kept filled with water in incubators housing infected mosquitoes. Moist cotton pads wrapped in paper towels (to collect laid eggs) were placed on top of mesh-secured mosquito buckets. To increase survival until harvesting, a large paper blanket was also placed over all buckets and moistened daily.

- burden of infection
    - microfilarial density: Standard practice for infections of this scale is to infect mosquitoes with 3,500 microfilariae/ml, although an estimated range of 3,000-5,000 mf/ml based on periodic Knott's or modified Knott's tests of the microfilaremic dog/s is often used to refrain from performing time-consuming microfilarial counts before each infection. To minimize variability, I performed microfilarial counts before every infection using methods described below. Blood was diluted with water and held at room

temperature for 20 mins to allow cells to lyse, facilitating microfilaria counts.

- ingested dose: In addition to microfilarial density, dose is dependent on blood meal volume and, therefore, mosquito size. Average ingested microfilariae was determined from midgut dissections immediately after mosquito infection, and was demonstrated to correlate with my counts of microfilarial density and mosquito size (Figure B2).

- intra-strain variation of vector competence: After multiple experimental infections of my *Ae. aegypti* Liverpool and *Ae. albopictus* NJ strains, I obtained *Ae. aegypti* Liverpool strain egg papers from Cheri-Hill Kennel & Supply Inc. (Stanwood, MI). *Dirofilaria immitis* infections have produced mature larvae in this strain in all experiments since. This colony was used by Cheri-Hill for maintenance of *D. immitis,* and it is assumed that inadvertent selection has improved their vector competence. Selection for *D. immitis* competence has been demonstrated in the literature (Nayar et al. 1988, Bradley et al. 1990).

Conclusions

Optimization of infection and harvesting informed my protocol below. Mosquito mortality leading up to detection of L3 infective stage larvae is an estimated 50%. Mortality can

be reduced by infecting with lower doses (Russell and Geary 1996), but this would increase the number of mosquitoes needed to harvest a target number of L3 *D. immitis* and, therefore, rearing space and labor requirements. I suggest, based on results from Chapter 3, that harvesting take place at the time of peak detection in mosquito heads, which may be a few days after accumulation of 130 HDUs or 3120 HDUhours (Chapter 3). Delaying harvesting past the peak will reduce the number of surviving infected mosquitoes and harvesting efficiency.

Methods

Mosquito rearing Mosquitoes were maintained in an environmental chamber set to 29˚C, 90% RH and 10 h L: 10 h D photo-regime with 2 h periods of dusk and dawn. Adults were held in a 30-cm^3 screened cage and provided with 20% sucrose ad libitum. Two- to five-week-old chicks were offered twice to three times a week for 20 mins at each blood feeding.

Eggs were collected on paper towels lining oviposition vessels placed inside the cage. Papers with eggs were air dried for 24 h and stored at 100% humidity for at least seven days and up to a month before hatching. Egg papers were soaked in a flask filled with 750 ml of water for 30 min before applying vacuum pressure for 45 min for hatching. Vacuum pressure was released and 30 mg of larval diet added. Larvae were held overnight in the flask and transferred into trays the next day.

Larvae were transferred to 3.9-liter plastic trays (21 by 21 by 7 cm) containing 1 L of purified water and secured with mesh tops. 200 larvae were added to each tray. Larval diet (Hikari® Cichlid Gold Large Pellet; Kyorin Co. Ltd. Himeji, Japan) was added to each tray according to the following regime (McLean-Cooper et al. 2008): day 1, 30 mg (ground fish

pellet); day 2, 30 mg (ground fish pellet); day 3, 582 mg (3 whole pellets and ground pellets). Synchronization of pupation and eclosion were analyzed as well as wing length (as a proxy for body size) to assure suitability of this diet regime.

Handling of microfilaremic blood Microfilaremic and non-infected blood were supplied in heparinized collection tubes by Dr. Byron Blagburn, Department of Pathobiology, College of Veterinary Medicine, Auburn University, Auburn, AL. Microfilaremic blood from tubes was pooled together before use.

Determining microfilarial density Microfilarial density of infected blood samples was determined immediately prior to each mosquito feeding. Microfilariae in 3 x 20 µl aliquots of blood were counted from the pooled blood sample. Counting was performed using a modified method based on Theis et al. (2000). Briefly, each 20 µl aliquot of infected blood was diluted in 40 µl of water and held at room temperature for 20 mins. Diluted aliquots of blood were each spread evenly over three microscope slides for scanning under a phase contrast microscope at 100X. Only moving microfilariae were counted. After determining the density of live microfilariae, heartworm-negative dog blood was added to attain the desired microfilarial density of 3500 microfilariae/ml.

Feeding/Infecting mosquitoes Cups holding an estimated 200 pupae were placed inside 7L plastic containers secured with mesh in which they were allowed to eclose. Moistened sugar cubes were provided on tissue over the mesh lid. Sugar and water were removed 15-20 h before offering microfilaremic blood. On the day of infection, mosquitoes were fed microfilaremic blood according to the methods of Lai et al. (2000) with the following

modifications. Known densities of microfilariae in heparinized dog blood were added to glass feeders secured at the base with washed hog casing (Syracuse Casing Company, Syracuse, NY). Blood was warmed in the feeder apparatus using a circulating water bath set at 37˚C (Harrington et al. 2001). Feeders were placed on mesh bucket lids, and mosquitoes were allowed to feed on infected blood for 30-60 min. A plastic disposable 3 ml pipette was used to mix blood within the feeders every 4-7 min to prevent microfilariae from settling unevenly on the feeding membrane (Kartman 1953). After feeding, mosquitoes were cold-immobilized for sorting. Fully engorged female mosquitoes were placed 200 per 7L bucket; less engorged females, unfed females, and males were discarded. All mosquitoes used in the study were infected on 8/28/13.

Blood fed females were maintained in buckets as described above and held at 90% RH and 29°C.

Harvesting of L3 stage *D. immitis* Preliminary experiments demonstrated that L3 stage *D. immitis* could be harvested after 15 days at 29˚C. Dissection of chilled mosquitoes was performed on glass slides in droplets of RPMI 1640 medium with added antibiotics as provided by Dr. Dwight D. Bowman, Department of Microbiology & Immunology, College of Veterinary Medicine, Cornell University, Ithaca, NY. Mosquitoes were decapitated and heads placed in RPMI drops in groups of about 7. *Dirofilaria immitis* L3 stage larvae observed escaping the heads into the droplet were hooked with dissection tools—minuten pins mounted into glass Pasteur pipettes—and transferred to syringes containing RPMI medium with antibiotics added. Heads were further dissected and labia and proboscises were separated to allow more larvae to

escape into the solution. Harvesting was performed as described from 0700-1600 hrs on September 12, 2012 producing a total of six syringes of 80 L3 stage larvae each. Syringes were kept on ice for the duration of *D. immitis* harvesting.

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APPENDIX C: L3 development rate descriptive statistics

Means and Medians for Figure 1 Time to Detection

	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
treatment	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
19°C	160.832	.447	159.955	161.709	-	-	-	-
19±9°C	152.912	2.093	148.809	157.015	132.601	6.122	120.603	144.599
Overall	161.987	1.411	159.221	164.753	178.899	5.952	167.232	190.566

a. Estimation is limited to the largest survival time if it is censored.

Means and Medians for Figure 2 Time to Detection

	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
treatment	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
19°C	160.832	.447	159.955	161.709	-	-	-	-
19±9°C	116.537	1.413	113.769	119.306	133.225	5.579	122.290	144.161
Overall	149.993	1.074	147.889	152.097	161.908	6.242	149.674	174.141

a. Estimation is limited to the largest survival time if it is censored.

Means and Medians for Figure 3 Time to Detection

	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
treatment	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
22°C	148.841	1.329	146.236	151.446	138.328	2.416	133.594	143.063
22±4°C	247.196	3.816	239.716	254.676	-	-	-	-
Overall	235.956	3.783	228.541	243.371				

a. Estimation is limited to the largest survival time if it is censored.

Means and Medians for Figure 4 Time to Detection

	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
treatment	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
26°C	190.397	6.191	178.262	202.532	-	-	-	-
26±4°C	273.110	3.608	266.038	280.182	-	-	-	-
Overall	267.686	3.569	260.691	274.681	-	-	-	-

a. Estimation is limited to the largest survival time if it is censored.

Means and Medians for Figure 5 Time to Detection

	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
treatment	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
19°C	161.511	.279	160.965	162.057	-	-	-	-
19±9°C	161.842	2.298	157.338	166.345	178.899	10.915	157.505	200.293
Overall	171.233	1.111	169.056	173.410	178.899	10.430	158.455	199.342

a. Estimation is limited to the largest survival time if it is censored.

Means and Medians for Figure 6 Time to Detection

	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
treatment	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
19°C	161.511	.279	160.965	162.057	-	-	-	-
19±9°C	123.066	1.508	120.110	126.023	133.225	7.267	118.982	147.469
Overall	154.952	.919	153.150	156.754	-	-	-	-

a. Estimation is limited to the largest survival time if it is censored.

Means and Medians for Figure 7 Time to Detection

	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
treatment	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
22°C	155.311	1.478	152.414	158.207	161.796	6.396	149.260	174.332
22±4°C	197.997	3.494	191.150	204.845	173.706	1.669	170.435	176.977
Overall	197.550	3.389	190.907	204.193	173.706	1.610	170.551	176.862

a. Estimation is limited to the largest survival time if it is censored.

Means and Medians for Figure 8 Time to Detection

	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
treatment	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
26°C	200.654	7.127	186.685	214.624	-	-	-	-
26±4°C	197.763	2.963	191.956	203.570	180.229	1.363	177.557	182.901
Overall	197.592	2.944	191.822	203.363	180.229	1.380	177.525	182.933

a. Estimation is limited to the largest survival time if it is censored.

APPENDIX D: Molecular methods and KAP questionnaire

DNA barcoding of blood meals Each 25 µl PCR tube contained 1.5 µl of template solution, 0.5 µl of 10 µM COI long Forward primer solution (Townzen et al. 2008), 0.5 µl of 10 µM COI long Reverse primer solution, 2.25 µl of 50 mM $MgCl_2$, 0.5 µl of 10 mM dNTP, 5 µl of 5x GoTaq Flexi buffer (Promega), 0.15 µl of 5 U/µl GoTaq Flexi DNA polymerase (Promega), and 14.6 µl of dH_2O (Gibco). Thermal cycling conditions consisted of 95°C for 5 min, 95°C for 30s, 50°C for 50 secs, 72°C for 1 min, and 72°C for 5 min. Cycles 2-4 were repeated an additional 39 times. PCR products were separated on an ethidium bromide-stained 1% agarose gel in 1X TBE buffer for 60 mins at 120V before being visualized and digitally photographed using a Multi Doc-It Digital Imaging System (UVP, Inc.). Blood meal extractions that did not produce clear bands were run with the COI short primer pair. Samples that did not amplify under these conditions were not analyzed further.

Samples with strong, clear bands were purified and prepared for sequencing as follows: 2 µl of ExoSAP-IT (Affymetrix, Inc.) were added to 5 µl of PCR product and incubated at 37 C for 15 mins and then 80 C for 15 mins. Each sample tube sequenced contained 1 µl of post-ExoSAP-IT PCR product, 1 µl of 10 µM of COI long Forward primer (COI short Forward primer was used for COI short primer PCR products), and 16 µl dH_2O (Gibco). Sequence data was viewed and edited in CodonCode Aligner (CodonCode Corporation, Dedham, MA). Sequences were entered into the Barcode of Life Database (BOLD, http://v3.boldsystems.org) and NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The threshold for positive matching was determined

based on quality control analysis with positive control samples and confirmation of species geographic ranges overlapping the collection sites.

Table D1: Name, sequence, target gene, amplicon size, and source are shown for each DNA barcoding and *D. immitis* primer set.

Primer name	Sequence (5'-3')	Target gene	Amplicon size	Source
COI long Forward	AAC CAC AAA GAC ATT GGC AC	Vertebrate cytochrome c oxidase I	663bp	Townzen et al., 2008
COI long Reverse	AAG AAT CAG AAT ARG TGT TG			
COI short Forward	GCA GGA ACA GGW TGA ACC G	Vertebrate cytochrome c oxidase I	324bp	Townzen et al., 2008
COI short Reverse	AAT CAG AAY AGG TGT TGG TAT AG			
DI COI Forward	AGT GTA GAG GGT CAG CCT GAG TTA	*D. immitis* cytochrome c oxidase I	203bp	Rishniw et al., 2006
DI COI Reverse	ACA GGC ACT GAC AAT ACC AAT			
LCO1490	GGT CAA CAA ATC ATA AAG ATA TTG G	Invertebrate cytochrome c oxidase I	~650bp	Folmer et al., 1994, Cywinska et al., 2006
HCO2198	TAA ACT TCA GGG TGA CCA AAA AAT CA			

Home address _____
○ Saint Augustine ○ Lake City
Resting Box Candidates:
Name_____
Number_____

Intro:

- *Researchers with Cornell University and University of Florida*
- *Doing heartworm and environment survey*
- *Short questionnaire*
- *Would you spare a few moments of your time?*
- ***Do you consent to this questionnaire?*** ○ **Yes** ○ **No**
- *Permission to examine yard for samples*

Pet Background

1. Do you own any pets?
   ○ Yes
   ○ No (Skip highlighted questions, go to 6)

2. Do you consider your pet(s) a part of your family?
   ○ Yes ○ No

3. May I know some info about your pet(s):

Dog / Cat	Name	Breed	Age	# Hrs Outdoors	Outdoors when?
					Dawn / Daytime Dusk / Night
					Dawn / Daytime Dusk / Night
					Dawn / Daytime Dusk / Night
					Dawn / Daytime Dusk / Night

4. *Dogs:* How frequently do you walk your dogs, and where?

Location	**Walks per week**
Dog park	
Local recreational park	
Local natural area	
Around neighborhood	
Let out in yard	
Other	

Heartworm Disease KAP Questionnaire - Version 6-28-2013
Please wait to ask questions until the end, when we will be able to explain everything. All identifiable information will be kept confidential, and your participation is completely voluntary.

We will now ask you a series of questions. Please answer each question as honestly as possible, and "I don't know" is always an acceptable response.

Please note answers below represent potential responses that may be checked by the interviewer to expedite the survey. To prevent potential bias, the respondent will not see these answers.

5. How long have you owned dogs or cats?

_____

Heartworm Knowledge

6. We will now list a number of diseases. Please let us know if you have heard of the disease, and if so, how much of a concern are they to you. Please use a scale from 0 to 5, 0 representing "not important", and 5 being "extremely important".

Disease	**Haven't heard**	**(0-5)**	**Heard, but can't rate**
West Nile			
Eastern Equine Encephalitis (Sleeping Sickness/Triple E)			
St. Louis Encephalitis			
Chikungunya			
Dengue			
Heartworm			

7. What animals can be infected with heartworm?

   ○ Dogs
   ○ Cats
   ○ Mammals
   ○ Don't know
   ○ Other _____

8. How do dogs become infected with heartworm?

   ○ Flea or tick bite
   ○ Contact with infected dog
   ○ Mosquito bite
   ○ Eating wild animals
   ○ Don't know
   ○ Other _____

9. Which months do you think animals are at risk of being infected with heartworm? (Best guess)

   ○ Jan. ○ Feb. ○ Mar. ○ Apr.
   ○ May ○ Jun. ○ Jul. ○ Aug.
   ○ Sept. ○ Oct. ○ Nov. ○ Dec.
   ○ All Year
   ○ Don't Know

10. What is your main source of information about heartworm?
○ Friend ○ Newspaper ○ None
○ Family member ○ Internet ○ Don't Know
○ Veterinarian (fill 11 = yes) ○ Other _____
○ TV/radio

Vet and Preventative Practices

Pet owners only:

11. Has your veterinarian informed you about heartworm?
○ Yes ○ Don't Know
○ No

a. How did your veterinarian present the information?
○ Conversation ○ Don't know
○ Pamphlet ○ Other _____
○ Website
_____

12. How often do you take your pets to the vet?
○ Once a year ○ Never
○ Every six months ○ Not sure
○ Only when necessary

13. Is your pet on heartworm preventative? ○ YES ○ NO

If YES:

a. Who gives the preventative medication?
○ Same person each time ○ Don't know
○ Whoever remembers ○ Other _____

b. What is the name of the preventative you use?
_____

c. Where do you buy refills of your medication?
○ Veterinarian ○ Don't know
○ Pet store ○ Other _____

d. How closely do you stick to the schedule?
○ Same day per month ○ Don't know
○ Same week per month ○ Other _____

e. How do you remind yourself to give the medication?
○ Note paper calendar ○ Don't know
○ Memory ○ Other _____
○ Electronic reminder

f. If a dose is missed, when is the next given?
○ As soon as possible ○ Don't know
○ Start of next month ○ Other _____

g. How many months of the year do you give heartworm preventative to your pets?

NOT 12 _____ months *12*

i. What is the last month that heartworm preventative is given each year?
_____

ii. How do you decide when to end treatment?
○ Vet recommends ○ Don't know
○ End mosq. season ○ Other _____

iii. What is the reason for not using heartworm preventative year-round?
○ Vet recommends ○ Don't know
○ End mosq. season ○ Other _____
○ Cooler weather
○ Cost
○ No need

If NO:

h. What is the reason for not giving preventative?
○ Cost ○ Don't know
○ No need ○ Other _____
○ Never considered it

Ask non-pet owners too:

14. If your dog or cat were infected with heartworm and not treated for it, how serious do you think it would be? *Please use a scale from 0 to 5, where 0 means not important and 5 means extremely important.*
_____

15. If your dog or cat were infected with heartworm, what do you estimate the cost of treatment would be?
$_____

16. If your dog or cat were infected with heartworm, would you be willing to spend:
○ Up to $100
○ Up to $500
○ Up to $1000
○ Over $1500

17. When it comes to the topic of heartworm, I'm likely to go out of my way to get more information.
○ Strongly disagree ○ Don't know
○ Disagree
○ Agree
○ Strongly agree

18. If I wanted to get more information about heartworm, I would know where to go.
○ Strongly disagree ○ Don't know
○ Disagree
○ Agree
○ Strongly agree

19. If I wanted to get more information about heartworm, I would know which questions to ask.
○ Strongly disagree ○ Don't know
○ Disagree
○ Agree
○ Strongly agree

APPENDIX E: Footnoted references

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